



The effect of disinfectants on *Listeria monocytogenes* – phenotypic, physiological and genetic response

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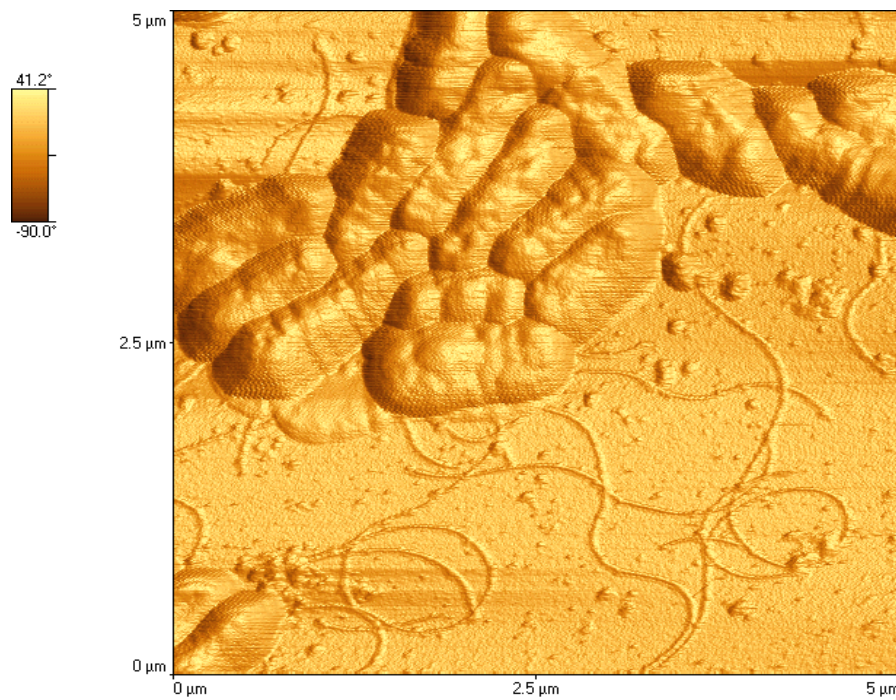
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Ph.D. thesis

**The effect of disinfectants on *Listeria monocytogenes*
– phenotypic, physiological and genetic response**



By
Vicky Gaedt Kastbjerg

April, 2009

The effect of disinfectants on *Listeria monocytogenes* – phenotypic, physiological and genetic response

Ph.D thesis
by
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April, 2009

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National Institute of Aquatic Resources
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Frontpage image:

Atomic Force Microscope image of *Listeria monocytogenes* Scott A
grown without NaCl spotted on silica. Scan size: 5x5 µm.

Preface

The work presented in this thesis is the results of a Ph.D. study following the Ph.D. program at the Technical University of Denmark. The Ph.D. study is part of an EU project entitled “Pathogen Combat: control and prevention of emerging and future pathogens at cellular and molecular level throughout the food chain”, which was financed by the European Commission within the VI Framework Program, contract no. 007081.

The Ph.D. student Vicky Kastbjerg has been enrolled at the Technical University of Denmark from the 1st April 2006 to 30th April 2009. The work has been carried out at:

- Technical University of Denmark, The National Institute of Aquatic Resources, DK-2800 Kgs. Lyngby, Denmark.
- Manchester Metropolitan University, Division of Biology, Manchester, M1 5GD, United Kingdom (1 week stay)
- University of Copenhagen, Faculty of Life Sciences, Department of Food Science, DK-1870 Frederiksberg C, Denmark (5 months stay)
- University of Copenhagen, Faculty of Life Sciences, Department of Veterinary Disease Biology, DK-1870 Frederiksberg C, Denmark (5 months stay)

Supervisors were Professor Lone Gram (National Institute of Aquatic Resources) and senior research scientist Birte Fonnesbech Vogel (National Institute of Aquatic Resources).

The thesis is based on the following papers including a report (not intended for publication):

Report

Vicky G. Kastbjerg and Kathryn A. Whitehead (2007). Use of atomic force microscope to determine the ease of bacterial removal for a food processing persistent strain and a type strain of *Listeria monocytogenes*. Not intended for publication.

Paper 1;

Vicky G. Kastbjerg and Lone Gram (2009). Model systems allowing quantification of sensitivity to disinfectants and comparison of disinfectant susceptibility of persistent and presumed nonpersistent *Listeria monocytogenes*. *Journal of Applied Microbiology* **106** 1667-1681.

Paper 2:

Vicky G. Kastbjerg, Dennis S. Nielsen, Nils Arneborg and Lone Gram (2009). Response at the single cell and population level of *Listeria monocytogenes* to disinfection stress monitored by measurements of intracellular pH and viable counts. *Applied and Environmental Microbiology*. Accepted

Paper 3:

Vicky G. Kastbjerg, Marianne H. Larsen, Lone Gram and Hanne Ingmer (2009). Sublethal concentrations of common disinfectants influence virulence gene expression in *Listeria monocytogenes*. *Applied and Environmental Microbiology*. Submitted

Vicky Gaedt Kastbjerg
April, 2009

Summary

The effect of disinfectants on *Listeria monocytogenes* – phenotypic, physiological and genetic response.

The incidences of food-borne disease are rising in EU for some pathogens despite many initiatives. Breaking the transmission of food-borne pathogenic bacteria from the processing environment through the food product to the consumer, together with controlling growth of bacteria in the food product, are of prime importance in reducing the number of food-borne illnesses. This is especially critical when producing Ready-to-eat (RTE) food products where the food is not exposed to a heating step before consumption. A key point in preventing contamination is the control of hygiene at the food processing plant level, and this is primarily obtained by efficient cleaning and especially disinfection, which is regarded as a critical step in eliminating spoilage and pathogenic bacteria in the food processing environment.

Listeria monocytogenes must be considered as one of the most important food-borne pathogens. This is due to several factors: the ubiquitous nature of *L. monocytogenes*, the ability of this bacterium to survive and grow in many types of food products, and the serious nature of the listeriosis infection. One of the big hurdles in controlling this pathogen is the remarkable ability of specific molecular sub-types to persist for years in the food processing environment. So far no specific trait has been identified that could explain this ability.

Several hypotheses have been proposed to explain the persistence of specific molecular subtypes of *L. monocytogenes* including enhanced adhesion to surfaces, enhanced tolerance to drying or increased resistance or tolerance to the chemical disinfectants used. The latter could be due to a general decreased susceptibility of the persistent sub-types or due to the presence of a less susceptible sub-population of bacterial cells during disinfection of the persistent sub-types. These hypotheses are addressed in the present thesis.

The expression of the virulence genes in *L. monocytogenes* are tightly regulated by the master regulator PrfA, (positive regulatory factor A), and are affected by environmental conditions including food related stress factors. In the food processing environment, the

disinfectants are used to kill the bacteria. However, the disinfection processes may not always be adequately performed leading to exposure of *L. monocytogenes* to sub-lethal concentrations. So far, it is not known how disinfectants in sub-lethal concentrations affect the expression of virulence genes in *L. monocytogenes*. A modulation of virulence gene expression by disinfectants may have impact on virulence or other biological properties of the bacterium.

The purpose of the present Ph.D.-study was to determine how disinfectants affect strains of *L. monocytogenes*. This has been studied on a phenotypic level to determine if different strains of *L. monocytogenes* including persistent and presumed non-persistent strains, respond differently to these compounds and if so, if this could explain the persistence of specific sub-types. Also, the effect of disinfectants has been studied on a physiological level by measuring the intracellular pH (pHi) as a stress measurement on single-cell level in order to determine if any sub-populations are detected during disinfection of a persistent strain of *L. monocytogenes*. Finally, the effect of disinfectants has been studied on a genetic level in order to determine how sub-lethal concentrations of these compounds affect virulence gene expression in *L. monocytogenes*.

Work in this thesis demonstrated that *L. monocytogenes* survives drying very well when protected by organic material. This was found as a general trait for the different strains of *L. monocytogenes* and not unique to persistent types. Furthermore, initial studies with atomic force microscopy (AFM) indicated no difference in adhesion strength between a persistent and a presumed non-persistent strain of *L. monocytogenes*.

We found differences in susceptibility between strains when evaluating the sensitivity to disinfectants of several strains of *L. monocytogenes* representing different origin both as planktonic, and spot inoculated and dried bacteria. However, the hypothesis that persistence is caused by increased tolerance to disinfectants could not be verified by the present study. A disinfectant based on peracetic acid caused a pronounced and homogenous decrease in pHi of a persistent sub-type, when studied on single cell level. Hence, it seems unlikely that specific molecular sub-types persist due to a sub-population with decreased susceptibility or resistance to disinfectants. In the present thesis, no specific trait for explaining persistence was identified and hence, more studies are needed to elucidate this riddle.

It was demonstrated that pre-growth with 5% NaCl increased the tolerance of planktonic *L. monocytogenes* cells to a peracetic disinfectant but not to a QAC disinfectant. Furthermore,

the presence of NaCl protected *L. monocytogenes* through drying, but not through disinfection of attached bacteria. Hence, it is not clear if differences in pre-growth could affect sensitivity of strains to disinfection procedures in the industry.

L. monocytogenes cells was found equally sensitive to disinfectants *per se* whether attached or suspended when calibrating model systems to include equal organic material and equal volumes and concentrations of disinfectants. Hence, the higher concentrations needed for eliminating attached bacterial cells as compared to planktonic cells must be due to protection from organic material, decreased possibility for the disinfectant to penetrate the cell, or cell changes only present when the cell is attached.

Sub-lethal concentrations of a peracetic disinfectant that did not affect viability of *L. monocytogenes* did stress the bacterial cells as measured by a decrease in pH_i. Also, sub-lethal concentrations of disinfectants affects expression of virulence genes in *L. monocytogenes* as determined with an agar-based assay and confirmed with Northern blot analysis. Further studies are needed to determine the phenotypic outcome and the possible effect on virulence of disinfectants. It is also relevant to study if exposure of *L. monocytogenes* to sub-lethal concentrations of disinfectants alters its resistance or tolerance to other stress factors, such as food preservations compounds or antibiotics.

Resumé (in Danish)

Effekten af desinfektionsmidler over *Listeria monocytogenes* – fænotypisk, fysiologisk og gentisk repons.

Der er de seneste år set stigninger i antallet af fødevarebårne sygdomme i EU for nogle af de patogene bakterier på trods af forskellige initiativer. De to vigtigste metoder til at reducere antallet af infektionstilfælde forårsaget af fødevarebårne patogene bakterier er at få brudt overførslen af fødevarebårne patogene bakterier fra produktionsmiljøet gennem fødevaren til forbrugeren og at kontrollere bakterievæksten i fødevaren. Dette er især kritisk ved produktion af de såkaldte spiseklare fødevarer, som indtages uden forudgående varmebehandling. For at forhindre kontaminering af fødevarer er det vigtigt med en god hygiejne i det fødevareproducerende anlæg, og dette opnås primært ved en effektiv rengøring og især desinfektionsproces, der betragtes som et kritisk punkt i at eliminere fordærvelses- og sygdomsfremkaldende bakterier i fødevareproduktionsmiljøet.

Listeria monocytogenes kan betragtes som en af de vigtigste fødevarebårne patogene bakterier. Det skyldes forskellige faktorer såsom dens ubikvitære natur, dens evne til at overleve og vokse i mange typer fødevarer, samt listeriainfektionens alvorlige natur. En af de store udfordringer i at kunne kontrollere denne patogene bakterie er den bemærkelsesværdige evne, som specifikke molekulære subtyper har, til at persistere i produktionsmiljøet. Der er indtil videre ikke blevet identificeret noget bestemt karakteristika hos disse sub-typer, som kan forklare deres evne til at persistere.

Der er blevet fremsat forskellige hypoteser, som kan forklare, hvorfor disse specifikke molekulære sub-typer kan persistere. Hypoteserne foreslår bl.a., at persistens kan skyldes en øget adhæsion til overflader, en øget tolerance overfor udtørring samt en øget resistens eller tolerance overfor de desinfektionsmidler, som anvendes i fødevareindustrien. Sidstnævnte kan enten forårsages af en generel nedsat følsomhed overfor desinfektionsmidler hos disse specifikke sub-typer eller tilstedeværelsen af en mindre følsom sub-population af bakterie celler under desinfektionen af en persisterende sub-type. Disse hypoteser er alle undersøgt i nærværende Ph.d. studie.

Hos *L. monocytogenes* er ekspressionen af adskillige virulensgener reguleret af PrfA (positiv regulator faktor A) og endvidere påvirket af fysiske-kemiske faktorer i omgivelserne, hvilket også inkluderer fødevare relateret stress. I fødevareindustrien bliver desinfektionsmidler anvendt med det formål at dræbe bakterier. Dog bliver desinfektionsprocessen ikke altid udført tilstrækkeligt, hvilket kan medføre, at *L. monocytogenes* bliver udsat for sub-letale koncentrationer. Det vides ikke, hvordan desinfektionsmidler i sub-letale koncentrationer påvirker ekspressionen af virulensgener i *L. monocytogenes*. En indvirkning af desinfektionsmidler på ekspressionen af virulensgener kan have betydning for virulensen eller andre biologiske egenskaber hos bakterien.

Formålet med nærværende Ph.d. studie har været at bestemme, hvordan desinfektionsmidler påvirker *L. monocytogenes* stammer. Dette er blevet studeret på fænotypisk niveau for at undersøge, om forskellige stammer af *L. monocytogenes*, inklusiv persisterende og formodede ikke-persisterende stammer, responderer forskelligt til disse produkter, og i så fald om det kan forklare disse specifikke subtypers evne til at persistere. Effekten af desinfektionsmidler er også blevet undersøgt på fysiologisk niveau ved at måle det intracellulære pH (pHi), som et mål for stress, på enkelt-celle niveau for at bestemme, om der kan detekteres sub-populationer under desinfektionen af en persisterende stamme af *L. monocytogenes*. Slutteligt er effekten af desinfektionsmidler blevet undersøgt på genetisk niveau for at bestemme, hvordan sub-letale koncentrationer af disse produkter indvirker på ekspressionen af virulensgener i *L. monocytogenes*.

Arbejde i denne afhandling har vist, at *L. monocytogenes* overlever udtørring i højt niveau, når den beskyttes af organisk materiale. Dette var dog en generel egenskab for de forskellige *L. monocytogenes* stammer, der blev undersøgt og ikke unikt for de persisterende stammer. Desuden indikerede indledende studier med atomar kraftmikroskopi (AFM), at der ikke var forskel mellem persisterende og formodede ikke-persisterende stammer af *L. monocytogenes* i deres adhæsions styrke.

Forskellige stammer af *L. monocytogenes* udviste forskellig følsomhed overfor desinfektionsmidler, både som planktoniske bakterier, og som spottede og tørrede bakterier. Hypotesen om, at persistens skyldes en øget tolerance overfor desinfektionsmidler kunne dog ikke verificeres i dette studie. Et desinfektionsmiddel baseret på pereddikesyre forårsagede et markant og homogent fald i pHi hos en persisterende sub-type ved studier på enkelt-celle niveau. Det virker derfor ikke sandsynligt, at specifikke molekulære sub-typer af *L. monocytogenes* er i stand til at

persistere grundet en sub-population med nedsat følsomhed eller resistens overfor desinfektionsmidler. Det er således fortsat nødvendigt med flere studier for at kunne identificere karakteristika hos disse sub-typer, som kan forklare deres evne til at persistere.

Vi har i denne afhandling demonstreret, at forudgående dyrkning med 5% NaCl øgede tolerancen af planktoniske *L. monocytogenes* celler overfor et pereddikesyre-holdigt desinfektionsmiddel, men ikke overfor et desinfektionsmiddel baseret på kvarternære ammonium forbindelser (QAC). Endvidere beskyttede tilstedeværelsen af NaCl *L. monocytogenes* under udtørring, men ikke under den efterfølgende desinfektion af fasthæftede bakterier. Det er således ikke klart, om forskelle i de forudgående vækstforhold har betydning for stammernes følsomhed overfor desinfektionsprocedurerne i industrien.

L. monocytogenes celler var lige følsomme overfor desinfektionsmidler *per se*, uanset om de var fasthæftede eller opløst, når modelsystemerne blev kalibreret, så de indeholdt de samme mængder af organisk materiale, og samme volumen og koncentration af desinfektionsmidler. Derfor må den højere koncentration af desinfektionsmiddel, som er nødvendig for at eliminere fasthæftede bakterie celler sammenlignet med planktoniske celler, skyldes andre faktorer såsom en beskyttelse af organisk materiale, nedsat mulighed for desinfektionsmidlet i at trænge ind i cellen eller celle ændringer, som kun er til stede, når cellerne er fasthæftede.

Sub-letale koncentrationer af et pereddikesyre-holdigt desinfektionsmiddel, som ikke havde nogen effekt på levedygtigheden af *L. monocytogenes*, havde en stress effekt på bakterie cellen, når pH_i blev bestemt. Ligeledes havde sub-letale koncentrationer af desinfektionsmiddel en indvirkning på ekspressionen af virulensgener hos *L. monocytogenes* bestemt med et agar-baseret assay og bekræftet med Northern blot analyse. Flere studier er nødvendige for at bestemme om desinfektionsmidler har indvirkningen på fænotypen og virulens hos bakterien. Det er endvidere relevant at undersøge om udsættelsen af *L. monocytogenes* for sub-letale koncentrationer af desinfektionsmidler ændrer dens resistens eller tolerance overfor andre stress-faktorer som konserveringsmidler eller antibiotika.

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Report

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1 Introduction

Eating food, preferably daily, is a prerequisite for mankind's existence, and in Western society, we mainly consume food that is processed on industrial scale. The mass production of food commodities is a continuous challenge to the industry with respect to food safety, which is of fundamental importance to prevent food borne diseases. There are three main types of food contaminants: microbiological, chemical and physical (Notermans and Powell, 2005). The vast majority of outbreaks of food-related illness are due to microbial pathogens rather than chemical or physical contaminants. Hence, pathogenic micro-organisms are a major safety concern for the food industry. The incidences of food borne disease are rising in EU for some pathogens despite many initiatives (EFSA, 2009). Breaking the transmission of food- borne pathogenic bacteria from the processing environment through the food product to the consumer, together with controlling the growth of bacteria in the food product, are of prime importance in reducing the number of food borne illnesses. This is especially critical when producing ready-to-eat (RTE) food products where the food is not exposed to a heating step before consumption. A key point in preventing contamination is the control of hygiene at the food processing plant level, and this is primarily obtained by efficient cleaning and especially disinfection, which is regarded as a critical step in eliminating spoilage and pathogenic bacteria in the food processing environment.

Disinfection in the food processing environment can be performed by chemical or physical methods. The chemical principle relying on disinfectants is the most widely used in the food industry. Their application includes disinfection of production plant and food containers, the control of microbial growth in food and drinks and the decontamination of carcasses. A range of the disinfectants used today became available during the development of the wood and later coal distilling industry, and the inorganic chemical industry (Table 1.1) (Hugo, 1991). Hence, these efficient and invaluable compounds have provided society with numerous benefits over more than 100 years through the control of micro-organisms in a variety of applications, both in clinical practice and in industrial settings including the food industry. The physical methods used for disinfection in the food processing environment include among others treatment with steam, ultraviolet light and irradiation.

Table 1.1 Antiseptics and disinfectants and their application into clinical practice (modified from Hugo, 1991; Russell, 2002).

Antiseptic/disinfectant	Discovery or first description	Introduction/application
Alcohols	BC	Early AD
Chlorine	1744	1847
Sodium hypochlorite	1789	1827
CRAs	1915	1916
Chlorine dioxide	1925	1946
Iodine	1812	1816
Iodophores	1949	1956
Formaldehyde	1867	1894
Glutaraldehyde	1904	1960s
Phenols		
Phenol	1834	1867
Cresol	1842	1890
Chlorocresol	1906	1908
Bisphenols	1906	1927
Triclosan	1906	Early 1970s
QACs	1856/1916	1933
Chlorhexidine	1946	1954
Peracetic acid		1955

Identification of agents involved in food-borne diseases began at the end of the 19th century with the clarification of the aetiology of botulism in humans (reviewed by Notermans and Powell, 2005). Later milestones include the recognition of *Clostridium perfringens* as a food borne pathogen in 1943, and *Bacillus cereus* in the 1950s. Awareness of human infections with *Listeria monocytogenes* spread throughout Europe and North America in the 1950s and food-borne transmission was suspected (Gray and Killinger, 1966), but it was not until the occurrence of an outbreak in Canada in 1981 that proper evidence was obtained for its food- borne transmission (Schlech et al., 1983).

L. monocytogenes is a human pathogenic bacterium that causes listeriosis, a severe infection (Farber and Peterkin, 1991). The incidence of human listeriosis is low, but it has a high case-fatality rate of around 30% (Vazquez-Boland et al., 2001). *L. monocytogenes* is mainly transmitted via RTE foods in which it can survive and grow at high NaCl concentrations, and low temperatures and oxygen content. *L. monocytogenes* is a ubiquitous bacterium and can be isolated from raw material for food production. However, the source of product contamination is more often the processing environment (Autio et al., 1999; Lundén et al., 2003b; Miettinen et al., 1999; Norton et al., 2001; Rørvik et al., 1995; Vogel et al., 2001a; Wulff et al., 2006), and the bacteria have a remarkable ability to persist for months or years in a food processing plant (Chasseignaux et al., 2001;

Giovannacci et al., 1999; Hoffmann et al., 2003; Holah et al., 2002; Keto-Timonen et al., 2007; Lundén et al., 2003b; Norton et al., 2001; Thimothe et al., 2004; Wulff et al., 2006). Persistence is defined as the repeated isolation of organisms of a specific molecular sub-type from the same processing environment (Keto-Timonen et al., 2007). So far no specific trait has been identified that can explain this ability to persist.

Several hypotheses have been proposed to explain the persistence of *L. monocytogenes* (Borucki et al., 2003; Holah et al., 2002; Lundén et al., 2000, 2008; Norwood and Gilmour, 1999; Wulff et al., 2006) including one stating that persistence could be due to an increased resistance or tolerance to the chemical disinfectants used. This could be due to a general decreased susceptibility of the persistent sub-types or due to the presence of a less susceptible sub-population of bacterial cells during disinfection of the persistent sub-types. These hypotheses are addressed in the present work.

During infection of the human host, *L. monocytogenes* invades and replicates in macrophages and other non-phagocytic cells. Inside these cells, *L. monocytogenes* has a well-described life cycle involving different virulence factors. The expression of the virulence genes in *L. monocytogenes* are tightly regulated by the master regulator, positive regulatory factor A, (PrfA), and are affected by environmental conditions including food related stress factors. In the food processing environment, the disinfectants are used to kill the bacteria. However, the disinfection processes may not always be adequately performed or presence of organic debris inactivates the disinfectants, leading to exposure of *L. monocytogenes* to sub-lethal concentrations. So far, it is not known how disinfectants in sub-lethal concentrations affect the expression of virulence genes in *L. monocytogenes*. Understanding such potential effects of disinfectants is important to elucidate if they may have impact on virulence or other biological properties of the bacterium.

The purpose of the present Ph.D.-study has been to determine how disinfectants affect strains of *L. monocytogenes*. This has been studied on a phenotypic level to determine if different strains of *L. monocytogenes*, including persistent and presumed non-persistent strains, respond differently to these compounds and if so, if this could explain the persistence of specific sub-types (Kastbjerg and Gram, 2009). Also, the effect of disinfectants has been studied on a physiological level by measuring the intracellular pH as a stress measurement on single-cell level in order to determine if any sub-populations are detected during disinfection of a persistent strain of *L. monocytogenes* (Kastbjerg et al., 2009b). This has been done to address the hypothesis that persistence could be due to the presence of a less susceptible sub-population. Finally, the effect of disinfectants has been

studied on a genetic level to determine how sub-lethal concentrations of these compounds affect virulence gene expression in *L. monocytogenes* (Kastbjerg et al., 2009a). A potential modulation of virulence gene expression by disinfectants may have impact on virulence or other biological properties of the bacterium.

This thesis reviews *L. monocytogenes* as a food-borne human pathogen (chapter 2) and describes hypotheses proposed and the studies performed to elucidate the persistence trait of this bacterium (chapter 3). The thesis also includes a review on the effect of chemical disinfection of *L. monocytogenes* performed on strain level (chapter 4). The methods to determine and the response of *L. monocytogenes* to stress on single cells level are described in chapter 5. Chapter 6 describes virulence regulation in *L. monocytogenes* including the regulation by PrfA and physico-chemical factors.

2 *Listeria monocytogenes* – a food-borne human pathogen

Listeria monocytogenes was discovered by EGD Murray in 1924 following an epidemic affecting rabbits and guinea pigs in animal care houses in Cambridge (Murray et al., 1926). The EGD strain used in many studies and in this thesis is a isolate from here. For many years, clinical *Listeria* isolates were a laboratory rarity, and the epidemiology of the disease was an unresolved mystery. However, at the end of the 1970s and the start of the 1980s, the number of reports on *Listeria* isolations began to increase and in 1983, the first human listeriosis outbreak directly linked to the consumption of *Listeria* contaminated foodstuffs was reported (Schlech et al., 1983). From 1983 onwards, a series of epidemic outbreaks in humans in North America and Europe clearly established listeriosis as a severe food-borne infection (Farber and Peterkin, 1991), and thereby *L. monocytogenes* as a food-borne pathogen. This chapter address the characteristics of *L. monocytogenes*, and the niches where it can be found including the food types that have been associated with listeriosis outbreaks. Finally, the possibilities to control *L. monocytogenes* as a food borne pathogen are described. The listeriosis disease including symptoms, incidence and fatality rate is further described in chapter 6.

2.1 Characteristics of the organism

The genus *Listeria* consists of a group of Gram-positive bacteria of low G+C content closely related to *Bacillus* and *Staphylococcus* (Sallen et al., 1996). The genus *Listeria* includes six species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri* and *L. grayi* (Sallen et al., 1996). Two of the species, *L. monocytogenes* and *L. ivanovii*, are pathogenic. While *L.*

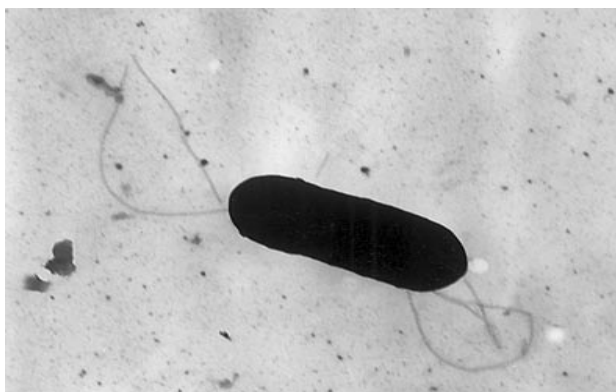


Fig 2.1. Electron micrograph of *Listeria monocytogenes* (copied from www.ext.vt.edu/pubs/seafood/600-070).

ivanovii is mainly pathogenic for animals, *L. monocytogenes* can infect humans, and domestic and wild animals (Seeliger and Jones, 1986). A third species, *L. seeligeri*, is considered avirulent, although it has been isolated from at least one case of human

listeriosis (Rocourt et al., 1986). Interestingly, it carries many of the listerial virulence genes, including that of the central virulence regulator PrfA (Gouin et al., 1994).

L. monocytogenes are nonsporeforming rods of 0.4 µm in diameter and 0.5 – 2 µm in length (Fig. 2.1). They are motile by means of peritrichous flagella when cultured below 25 °C (Seeliger and Jones, 1986). *L. monocytogenes* can grow over the temperature range of 1-45 °C with optimum between 30 °C and 37 °C. It grows over a pH range of pH 6 to pH 9, but dependent of acid type and temperature, *L. monocytogenes* can grow at pH as low as 4.0 (Martin and Fisher, 1999). *L. monocytogenes* grow in complex medium containing up to 10% (W/v) NaCl, but some strains can tolerate up to 20 % (W/v) NaCl (Seeliger and Jones, 1986). Since *L. monocytogenes* is facultative anaerobic (Seeliger and Jones, 1986), it can grow in aerobic modified atmosphere also with competitive organisms (Wimpfheimer et al., 1990). Temperature, pH, NaCl and oxygen content are parameters often adjusted to control bacterial growth in food products. However, since *L. monocytogenes* can grow at low temperatures and oxygen content and with high NaCl, this bacterium is very well equipped to survive these hurdles.

L. monocytogenes is classified into 13 different serovars based on O (somatic) and H (flagellar) antigens (Seeliger and Höhne, 1979; Seeliger and Jones, 1986). The 13 serovars are designated 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7. Although human listeriosis may be caused by all 13 serovars of *L. monocytogenes*, serovars 1/2a, 1/2b, 1/2c and 4b cause at least 95% of the cases (Farber and Peterkin, 1991; Doumith et al., 2004; Swaminathan and Gerner-Smidt, 2007). Among the outbreaks of invasive listeriosis, serovar 4b strains caused the majority of the outbreaks worldwide from 1980-2005, whereas strains of serovar 1/2 caused the majority of the non-invasive, gastrointestinal listeriosis outbreaks worldwide from 1993-2001 (Swaminathan and Gerner-Smidt, 2007). Among food isolates, serotype 1/2 is the most frequently found (Farber and Peterkin, 1991; Jacquet et al., 2002).

Genotypic analyses have also been used to group *L. monocytogenes*. Whereas multilocus enzyme electrophoresis (Piffaretti et al., 1989), pulsed-field gel electrophoresis (PFGE) (Brosch et al., 1994) and ribotyping (Graves et al., 1994) consistently grouped *L. monocytogenes* into two major sub-groups, virulence gene allelic analyses of three virulence genes (*actA*, *inlA*, *hly*) have grouped strains of *L. monocytogenes* into three groups, lineage I, II and III (Rasmussen et al., 1995; Wiedmann et al., 1997). The genetic lineages predicted the following serovar clusters when studying *L. monocytogenes* strains of nine serovars: lineage I, comprising serotypes 1/2b, 3b, 3c and 4b; lineage II,

comprising serotypes 1/2a, 1/2c and 3a, and lineage III comprising serotypes 4a and 4c (Nadon et al., 2001). Consequently, invasive listeriosis is primarily caused by lineage I strains, whereas lineage II strains are most frequently isolated from food.

2.2 Natural niches

L. monocytogenes is ubiquitous in the outdoor environment. It is thought to be widespread, being a saprophytic organism adapted to the plant-soil environment (Weis and Seeliger, 1975). The bacteria is widely present in plant, soil, sediments and surface water samples, and has also been found in sewage, human and animal faeces (MacGowan et al., 1994; Weis and Seeliger, 1975). Generally, the proportion of positive samples is low in the outdoor environment, between 0 and 6%, but studies indicate that prevalence increases with the degree of human activity (El Marrackchi et al., 2005; Hansen et al., 2006; MacGowan et al., 1994).

Animals are susceptible to listeriosis but can carry *L. monocytogenes* asymptotically. Faecal carriage of *L. monocytogenes* in livestock animals such as cattle, hens, sheep, pig and horses has been found with varying frequency around 5-15%, and in cattle up to 33% (Lyautey et al., 2007; Weber et al., 1995). Prevalence of *L. monocytogenes* has also been reported in wild life animals like deer, moose and birds (Hellström et al., 2008; Lyautey et al., 2007). Similarly, humans can be asymptomatic carriers of *L. monocytogenes*, but generally with prevalence of below 1% (MacGowan et al., 1994; Sauders et al., 2005).

2.3 *Listeria monocytogenes* in foods

The vehicle of infection by *L. monocytogenes* was unknown until the first food-borne outbreak of listeriosis in Canada in 1981. This outbreak involved 41 cases including both adults and perinatal infection due to *L. monocytogenes* serotype 4b, and provided an opportunity to examine the risk factors for the acquisition of this uncommon infection. A case-control survey was done and resulted in an association of coleslaw consumption with illness. The track was followed back to the farmer, who raised cabbage in fields fertilized by both composted and raw manure from a flock of sheep of which two had died of listeriosis in 1979 and 1981, respectively (Schlech et al., 1983). This outbreak was followed in the subsequent years by a series of epidemic outbreaks in humans in North America and Europe which established listeriosis as an important food-borne infection (reviewed by Farber and Peterkin, 1991).

Foods of different product categories have been implicated in outbreaks of listeriosis. These include meat products like pork tongue in jelly, sausage, pâté, sliced cold meat and

rillettes; dairy products like different types of cheeses, soft, semi-soft and mould-ripened including cheeses of raw milk, butter and ice cream; seafood products like gravad trout, cold-smoked rainbow trout, vacuum-packed fish products and shellfish; vegetables products like rice and corn salad, and coleslaw (Farber and Peterkin, 1991; Valk et al., 2005). Most of these are RTE products that are eaten without further cooking or reheating. Furthermore, these products are kept refrigerated, have a long shelf-life, and contains concentrations of salt and oxygen that *L. monocytogenes* benefits by. This gives *L. monocytogenes* the ability to grow in the products during storage.

The prevalence of *L. monocytogenes* in RTE foods in the US was generally determined to be 1.82% in 31,705 tested samples. The highest rate of positive samples were from seafood salads (4.7%) and smoked seafood (4.3%) (Gombas et al., 2003). The majority of positive samples had a contamination level of <10 cfu/g. However, a few samples had a contamination level of > 100 cfu/g and were from luncheon meats and smoked seafood. In a European survey of RTE products, the highest prevalence (18.2%) was found in smoked fish. Also, fishery products had the highest proportion of samples exceeding 100 cfu/g (2.2%) (EFSA, 2009). The minimal number of pathogenic *L. monocytogenes* cells which must be ingested to cause illness in either normal or susceptible individuals is not known. However, it has generally estimated to be >10³ cfu/g (FAO/WHO, 2004).

Although *L. monocytogenes* is a ubiquitous bacterium that can be present in raw materials for food production, several studies have shown that the immediate source of product contamination is more often the processing environment itself (Autio et al., 1999; Lundén et al., 2003b; Miettinen et al., 1999; Norton et al., 2001; Rørvik et al., 1995; Vogel et al., 2001a, Wulff et al., 2006). Mostly, the frequency of raw material samples containing *L. monocytogenes* is low, whereas the frequency of the bacteria rises in the food processing environmental samples and in samples taken from the products during the processing. For example Rørvik et al. (1995) found a higher frequency of samples containing *L. monocytogenes* from the environment than from the raw material both in a fish slaughterhouse and in a fish smokehouse. Also, the frequency of positive samples was lower for fish before filleting compared to samples taken from fish after filleting and further in the production process.

The use of molecular typing methods has given the ability to further sub-type the different isolates and survey their distribution in the production sites and more specifically to detect the contamination sources. Different typing methods have been used to track *L. monocytogenes* during processing: PFGE (Autio et al., 1999), ribotyping (Norton et al.,

2001), multilocus enzyme electrophoresis (Rørvik et al., 1995), random amplified polymorphic DNA (RAPD) (Vogel et al., 2001a); amplified fragment length polymorphism (AFLP) (Keto-Timonen et al., 2007) and PCR-REA (Giovannacci et al., 1999). The use of these methods has confirmed that the contamination of the final products in the processing plants is primarily due to the contamination during processing rather than contamination from the material, since the DNA types found in the final products also were found in many samples obtained from the processing environment, whereas the area with raw material harboured different strains (Autio et al., 1999; Norton et al., 2001; Vogel et al., 2001a; Wulff et al., 2006). However, the raw material cannot be excluded as a contamination source. Also, typing methods have been used to point out equipment, floors and drains as important contamination sources in the processing lines (Hoffmann et al., 2003; Miettinen et al., 1999; Norton et al., 2001; Keto-Timonen et al., 2007), and to demonstrate that specific sub-types are able to persist in the food processing environment for up to 10 years (Wulff et al., 2006). The persistence phenomenon will be further described in chapter 3.

2.4 Control of *Listeria monocytogenes*

Besides of being a risk to the consumer, the contamination of food-products with *L. monocytogenes* also has economic consequences both as medical costs and for the food manufacturer in cases of outbreaks. In 2000, the Economic Research Service of USDA (United States Department of Agriculture) estimated the food-borne illness cost in the US on the basis of the annual number of food-borne illnesses in 1998 for four major pathogens, including *L. monocytogenes*. They found that the total cost was \$6.9 Billion of which *L. monocytogenes* account for \$2.3 Billion for cases requiring hospitalization (Crutchfield and Roberts, 2000). Also, the food industry can have major expenses due to product contamination with *L. monocytogenes* as in cases with recall of products, factory closing, extraordinary cleanings, and compensation to infected people. This was seen lately in a large outbreak of *L. monocytogenes* in Canada in 2008 resulting in 57 cases with 22 deaths (Anonymous, 2009a). The outbreak were caused by meat products from a company that itself estimates their costs to be around \$43 million for recalls, lost sales and compensations to claimants (Anonymous, 2009b). Accordingly, there is a wide interest from different sides to control this bacterium.

Basically, there are two ways of controlling *L. monocytogenes* as a food-borne pathogen: by preventing contamination of, and by preventing growth in, the food product. Both are primarily in the hands of the food producers.

To prevent contamination of food-products with *L. monocytogenes* during the manufacturing processing, the following issues are important (Kornacki and Gurtler, 2007).

- The factory and equipment should be designed so they are easily cleaned, and cross-contamination to the product is minimal.
- The employees must have a thorough understanding of food hygiene
- The cleaning and sanitation must be effective
- Critical points in the process where contamination can occur should be identified and controlled
- The raw material must be of good microbiological quality

While careful compliance with the above mentioned issues might help reduce product contamination, they cannot guarantee products free of *L. monocytogenes*. Furthermore, *L. monocytogenes* is typically heterogeneously distributed and present at very low levels on contaminated products. Consequently, most procedures used for end-product testing will not detect the organism, and therefore they cannot be relied upon to ensure the safety of the products if subsequent growth can occur (ILSI, 2005). Therefore, preventing growth of *L. monocytogenes* in the food product is also important to reduce listeriosis.

A huge amount of work has been done to study different parameters and their influence on limiting growth of *L. monocytogenes* in different food products. Besides controlling the two important parameters, storage temperatures and times, also reformulation of foods, so they retard or do not support growth of *L. monocytogenes*, is intensively studied. Various interventions have been developed including biopreservation approaches (e.g. lactic acid bacteria, lytic bacteriophages and bacteriocins) (Jofré et al., 2007; Leverentz et al., 2003; Nilsson et al., 1999); application of plant extracts with antibacterial activity (e.g. spices, fruit extracts) (Apostolidis et al., 2008; Gutierrez et al., 2008; Hao et al., 1998) or chemical antimicrobial compounds (e.g. nitrite, salts of organic acids) (Glass et al., 2002; Glass et al., 2007; Seman et al., 2008). For fruit and vegetables, different decontamination technologies such as irradiation and sanitizing have been applied (Bari et al., 2005; Dhokane et al., 2006; Sy et al., 2005). Also, packing in a modified atmosphere in combination with other antimicrobial compounds has been shown to be effective (Nilsson et al., 1997; Mejlholm et al., 2005; Mejlholm et al., 2007). However, not all studies take sensory characteristics into account which is important, as the intervention should not alter the product for the worse.

In Europe, levels of *L. monocytogenes* in RTE food are regulated by commission regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs. The EU-regulation differentiates between products that are able and unable to support growth. In both cases the critical limit is 100 cfu/g, and it must be documented that this is not exceeded through the storage period. If this cannot be documented, *L. monocytogenes* should not be detected in the product samples (25 g) (EC, 2005).

2.5. Conclusions from chapter 2

Due to the ubiquitous nature of *L. monocytogenes*, the possibility of this bacterium to grow and survive in many types of food products, and the serious nature of listeriosis infection, *L. monocytogenes* has been manifested as an important food-borne pathogen. *L. monocytogenes* contaminates food products during processing, indicating that it has a remarkable ability to survive well in the food processing environment. Due to the pathogenic nature of *L. monocytogenes*, many interventions are tried in order to control this bacterium that continues to be a great challenge.

3 Persistence of *Listeria monocytogenes*

L. monocytogenes is a ubiquitous bacterium that can be present on raw materials for food production. However, the immediate source of product contamination is more often the processing environment itself. Molecular sub-typing has been used successfully to track the ecology of *L. monocytogenes* in food processing plants and to detect contamination sources. Furthermore, molecular sub-typing has demonstrated that specific strains of *L. monocytogenes* are able to persist in the food processing plants for months or years. This chapter address a definition of persistence and describes the origin of the persistent strains used in this thesis. Furthermore, it describes the hypotheses proposed to explain the persistence trait of *L. monocytogenes* and discuss the studies performed to elucidate this riddle.

3.1 Definition of persistence

L. monocytogenes has a remarkable ability to reside in different types of food processing plants. It has been repeatedly isolated from the production environment in dairy (Miettinen et al., 1999; Unnerstad et al., 1996), meat and poultry-processing (Chasseignaux et al., 2001; Giovannacci et al., 1999; Lundén et al., 2003b), RTE food processing (Holah et al., 2002; Keto-Timonen et al., 2007), and fish processing including both fish slaughterhouses and smokehouses (Hoffman et al., 2003; Norton et al., 2001, Thimothe et al., 2004; Wulff et al., 2006). Often, each of these processing plants had its own, unique contamination pattern of specific sub-types of *L. monocytogenes* with one or few molecular sub-types that dominated (Table 3.1). Some of the specific molecular sub-types can repeatedly be isolated over a longer time period from the same factory.

In this thesis, persistence refers to the ability to repeatedly isolate a specific molecular sub-type or strain in the same factory during an extended period of time; typically months or years (Keto-Timonen et al., 2007). Others denote a subtype as persistent if it has been isolated repeatedly from a product from the same factory (Norwood and Gilmour, 1999). On the other hand, the term presumed non-persistent are used rather than non-persistent in this thesis for describing sporadically isolated types of *L. monocytogenes* as they could in principle persist in other settings (Jensen et al., 2007; Kastbjerg and Gram, 2009).

3.2 The origin of the persistent strains used in this thesis






































This thesis includes work with four persistent strains of *L. monocytogenes* representing both lineage I and lineage II (Kastbjerg and Gram, 2009, Kastbjerg et al., 2009b). Two of these strains (N53-1 and La111) represent a specific molecular sub-type denoted RAPD type 9. Strains of this sub-type have been isolated from several different fish production sites and have been the persistent sub-type in several plants (Wulff et al., 2006). From a large study of eight fish processing plants, 213 strains were isolated and divided into 37 different RAPD-types (Table 3.1). Each plant had its own specific group of RAPD types; however, five sub-types were recovered from more than one smoke-and/or slaughterhouse including RAPD type 9. This specific RAPD type was the most commonly isolated (86 of 213 strains). It persisted in one smoke house for a 1.5 year period and clearly was the dominant strain. Also, it appeared to persist in three of the other processing environments (smokehouse 4 and slaughterhouse A and B) including production sites on Faeroe Island and products from Greenland, and it has been detected in fish processing for more than eight years (Vogel et al., 2001b; Wulff et al., 2006). Some of these processing plants have an inter-trade relationship, but some have never been in contact with each other. The two other persistent strains used have been identified as persistent in smoke houses for four years (La22) and one year (V518a), respectively (Vogel et al., 2001a). The ability of *L. monocytogenes* to persist correlates with the frequent isolation of *L. monocytogenes* from the processing environment itself. However, it also raises the concern that these persistent strains may cause repeated product contamination which in worst cases might lead to illness. Consequently, understanding the factor(s) that determines the persistence of these subtypes would be important for reducing contamination.

3.3 Why can specific sub-types of *Listeria monocytogenes* persist?

The persistence of specific sub-types of *L. monocytogenes* in different food processing environments seems not to be caused by a high prevalence of these specific sub-types in the outdoor environment, since the persistent RAPD type 9 strain was only isolated sporadically in water, fish, sediment and soil samples (Hansen et al., 2006). So far, this is the only study comparing molecular profiles of persistent strains with strains in the outdoor environment. However, if these strains are not brought into the production environment repeatedly, one could hypothesise that they must have a specific physiological trait that determines persistence of these certain subtype of *L. monocytogenes*. Consequently, several working hypotheses have been raised concerning the physical advantage that could explain the

Table 3.1. Distribution of *Listeria monocytogenes* RAPD types in smokehouses and slaughterhouses^a
(Copied from Wulff et al., 2006).

from Wani et al., 2000).

RAPD type		No. of <i>L. monocytogenes</i> strains of RAPD type at processing plant:								Total no. of strains
		Fish slaughterhouse				Smokehouse				
No.	Symbol	A	B	C	D	1	2	3	4	
5		3				1				4
9		30		18		3	25		10	86
30		5								5
31		2								2
32		1								1
33		1								1
7						4	1			6
34			1							5
35			5							5
36			1							1
37				7						7
38				1						1
39					5					5
40					1					1
41										1
42						1				1
44						4				4
45						8				8
57						5		11	12	28
46							1			1
47							1			1
48							1			1
49							1			1
52							1			1
53							2			2
54							2			2
55							5			5
6							1	17		18
58								2		2
59								1		1
60								1		1
61								1		1
62								1		1
63								1		1
64									4	4
65									1	1
66									1	1
X	X			3		5	2	11	4	25
Total		46	7	29	7	28	43	46	32	238

^a Strains were isolated during production and after cleaning and disinfection 1 to 3 months apart at each fish processing plant. The numbers of RAPD types found in more than one fish plant are in boldface. X, the number of *L. monocytogenes*-positive samples detected only by PCR and from which no strain were recovered

persistence trait (Borucki et al., 2003; Holah et al., 2002; Lundén et al., 2000, 2008; Norwood and Gilmour, 1999; Wulff et al., 2006):

- Enhanced tolerance to food stresses
- Enhanced adhesion and aggregation to surfaces
- Enhanced tolerance to drying
- Enhanced survival in co-culture
- Enhanced tolerance or resistance to cleaning and disinfection processes caused by a higher tolerance *per se* or by appearance of a more tolerant sub-population

Several of these hypotheses will be discussed in the following sections. The concern of the enhanced tolerance or resistance to disinfectants and the appearance of a more tolerant sub-population have been studied in this thesis and will be discussed in chapter 4 and 5, respectively.

3.3.1 Enhanced tolerance to food stresses

It has been hypothesised that differences in growth rate, either lower or higher, could explain the persistence of specific subtypes of *L. monocytogenes* (Holah et al., 2002; Wulff et al., 2006). A higher growth rate would enable the persistent sub-type to outcompete other sub-types in the processing environment as seen during the enrichment procedure where lineage II strains out-compete lineage I strains (Bruhn et al., 2005). On the other hand, a decreased growth rate could slow down the metabolic processes and thereby protect the bacteria against stresses (Balaban et al., 2004). However, the persistent strains had the same growth rate as the presumed non-persistent strains when grown in LB with 1% glucose with or without the addition of 5% NaCl at both 5 °C and 37 °C (Jensen et al., 2007). These studies were performed with the strains grown individually and one might speculate that growth in mixed culture may alter the picture, e.g. favouring the persistent sub-types in a co-culture set-up.

The ability to persist in the food processing environment could also be associated with a better tolerance to food related stress factors. Observed differences in acid and heat tolerance between *L. monocytogenes* strains (Casadei et al., 1998; Lianou et al., 2006) led to the hypothesis that acid or heat tolerance could be related to persistence (Lundén et al., 2008). Though variable between individual strains in general, the persistent strains did not show higher tolerance to these stresses as compared to other strains of *L. monocytogenes* (Lundén et al., 2008). However, a minor difference in acid tolerance was detected between persistent and presumed non-persistent strains after exclusion of exceptionally acid-susceptible non-persistent strains which distorted the normal distribution. A persistent sub-type was as sensitive to the processing steps in cold-smoked salmon production including brine and smoke as compared to two other strains of *L. monocytogenes*, a clinical strain and the EGD strain (Porsby et al., 2008). Hence, so far no systematic differences have indicated that decreased sensitivity to food-stresses could explain persistence.

3.3.2 Enhanced adhesion and aggregation to surfaces

L. monocytogenes can adhere to different kinds of surfaces found in the food processing environment like stainless steel, rubber, plastic, glass, PVC (Djordjevic et al., 2002; Mafu et al., 1990). During static conditions, *L. monocytogenes* forms an unstructured biofilm

composed of a few layers of cells covering the surface (Kalmakoff et al., 2001; Rieu et al., 2008a). No mushroom structures or other complex cellular structures are detected. The ability to adhere to surfaces could be different between persistent and presumed non-persistent strains, enabling the persistent strains to colonize the processing environment more efficiently.

Several studies have compared the surface adherence ability of persistent and presumed non-persistent isolates using different set-ups and different surfaces. Whilst some studies have concluded that persistent strains of *L. monocytogenes* adhere better to surfaces than presumed non-persistent strains (Borucki et al., 2003; Lundén et al., 2000; Norwood and Gilmour, 1999), others have found no relationship between environmentally persistent strains and their ability to adhere (Djordjevic et al., 2002; Jensen et al., 2007). The lack of overall agreement between the results could be explained by the use of different methods. However, Borucki et al. (2003), Djordjevic et al. (2002), and Jensen et al. (2007) used the same microtiter-assay but found different results in the comparison of adhesion and persistence. The different authors define the persistent strains differently. The persistent strains used by Borucki et al. (2003), and Norwood and Gilmour, (1999) have been isolated repeatedly from a food product from the same production site and not from the processing site itself. However, Lundén et al. (2000) used persistent strains isolated repeatedly from the same processing site for months or years as did Djordjevic et al. (2002) and Jensen et al. (2007). Hence, there is no clear evidence that differences in adherence capability can explain persistence.

The ability to adhere can be influenced by food stress factors such as NaCl and temperature. Interestingly, growth with 5% NaCl stimulated adhesion of *L. monocytogenes* and caused the bacteria to aggregate at 37 °C but not at 15 °C (Jensen et al., 2007). However, this was seen both for persistent and presumed non-persistent strains. Sublethal concentrations of ethanol and isopropanol increased attachment of *L. monocytogenes* at 30 °C and below (Gravesen et al., 2005). However, this might be a general trait for *L. monocytogenes* as the study was performed with three strains of *L. monocytogenes*, none of them characterized as persistent strains.

The ability to adhere is often determined as the biomass or cell numbers that adhere in a microtiter plate or on coupons, respectively, during growth. However, in this thesis it was hypothesised that differences in attachment strength could allow the persistent strains to colonize more efficiently (Kastbjerg and Whitehead, 2007). This was studied by atomic

force microscopy (AFM) (Fig 3.1) with two strains, a persistent RAPD type 9 strain (N53-1) and the type strain *L. monocytogenes* ScottA.

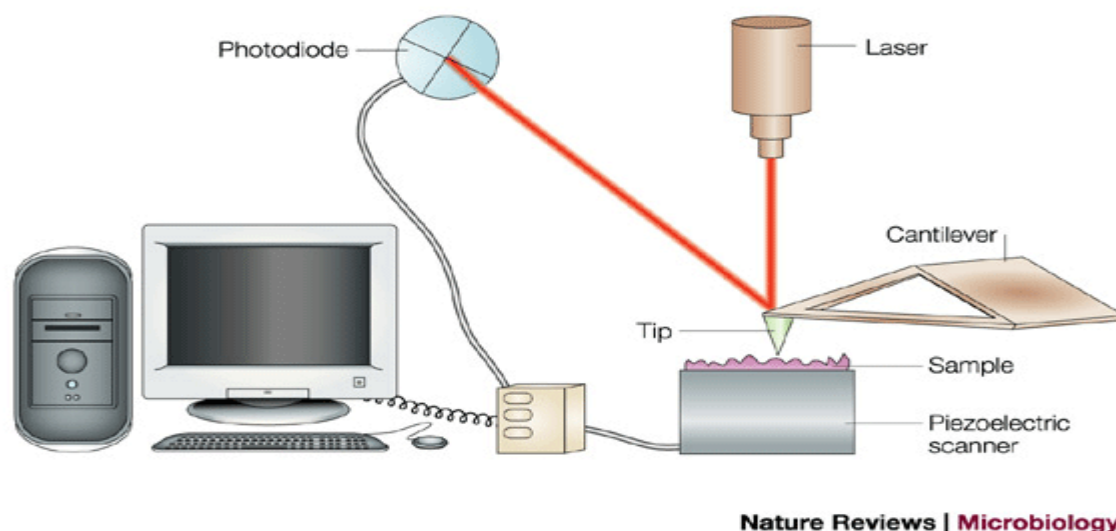


Fig 3.1. Atomic force microscopy (AFM) are based on the generation of images of surfaces by measuring the physical interaction between a sharp tip and the sample rather than by using an incident beam (light or electrons) as in classical microscopy. The main parts of an atomic force microscope are the sample stage, the cantilever and the optical detection system, which comprises a laser diode and a photodetector. The sample is moved relative to the cantilever in three dimensions using piezoelectric ceramics. The force interacting between the tip and the sample is monitored with piconewton (10^{-12} N) sensitivity, by attaching the tip to a soft cantilever, which acts as a spring, and measuring the bending (or deflection) of the cantilever. The cantilever deflection is usually detected by a laser beam focused on the free end of the cantilever and is reflected into a photodiode. AFM cantilevers and tips are usually made of silicon or silicon nitride using microfabrication techniques (copied from Dufrêne, 2004).

AFM can be used to determine the strength of bacterial attachment on a surface, since it can measure forces applied directly on cell-cell and cell-surface interactions, and the ease of removal of cells from a surface (Dufrêne, 2002). During scanning, the perpendicular force between the tip and the surface is kept low so that the cantilever and tip move in response to changes in surface topography. However, by deliberately increasing the perpendicular tip-surface force, the AFM-tip can be used to displace attached cells, and the number of removed cells after each scanning can be quantified together with the force used (Boyd et al., 2002; Whitehead et al., 2006).

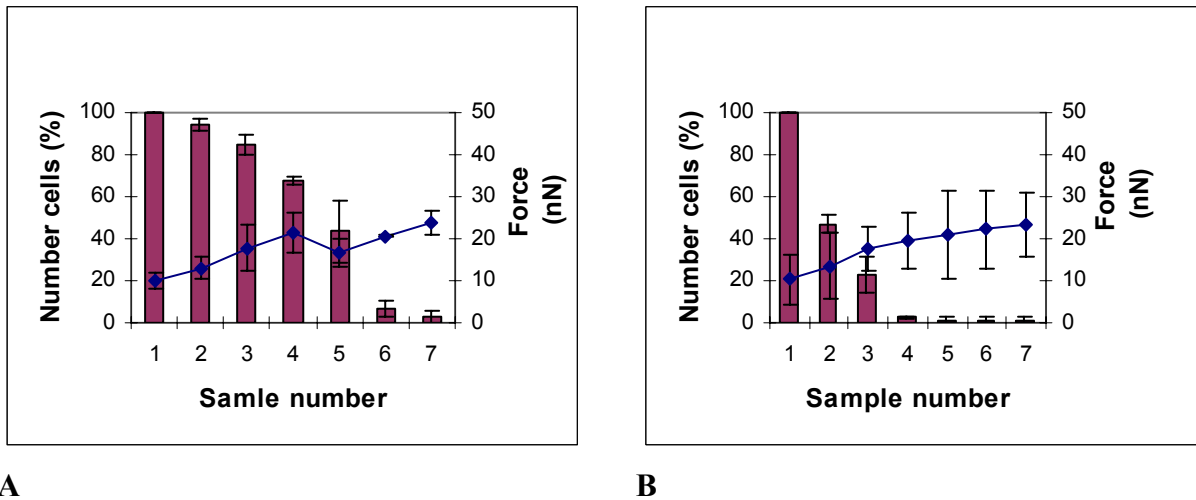


Fig 3.2. Removal of *Listeria monocytogenes* Scott A grown with NaCl (A) and without NaCl (B) at increasing perpendicular force over time spotted on SS illustrating that with increased force (line) a greater number of cells (columns) are removed from the substratum (Kastbjerg and Whitehead, 2007).

Listeria monocytogenes Scott A appeared to adhere more tightly to stainless steel when grown with NaCl, since the number of remaining cells decreased more rapidly for cells grown without NaCl as compared to cells grown with NaCl (Fig. 3.2). This difference in adhesion strength was not seen with the persistent *L. monocytogenes* N53-1. However, the latter comparison was only based on visual and not numerical comparisons.

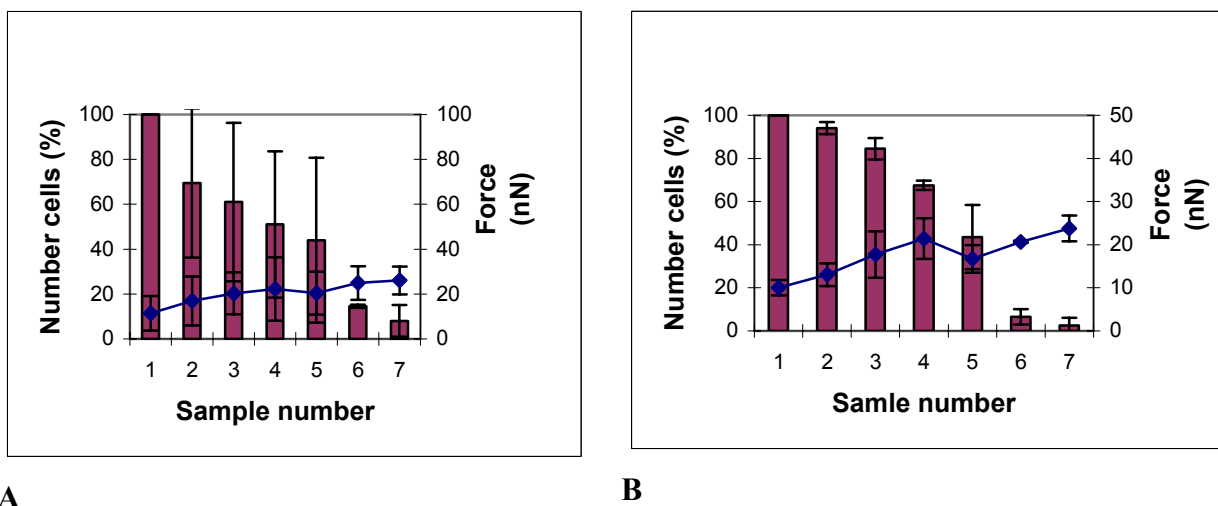


Fig 3.3. Removal of *Listeria monocytogenes* N53-1 (A) or *L. monocytogenes* Scott A (B) grown with NaCl at increasing perpendicular force over time spotted on SS illustrating that with increased force (line) a greater number of cells (columns) are removed from the substratum (Kastbjerg and Whitehead, 2007).

We found no difference in adhesion strength between the two strains *L. monocytogenes* Scott A and N53-1 grown with NaCl (Fig. 3.3). Minor differences were seen in the remaining cell number after scan number two, but due to the standard deviation for N53-1, this difference is not significant.

The results indicate that growth with NaCl increases the attachment strength, but that a persistent RAPD type 9 strain does not adhere better as compared to a type strain of *L. monocytogenes*. However, in general, the study must be repeated in order to confirm these observations. This includes scanning of more samples and standardization of the initial cell numbers in the scanning areas. AFM is a very sensitive method and has been used for detecting differences in attachment strength of different sized and shaped bacteria (Whitehead et al., 2006). Similarly, we found the method sensitive. Though sensitive, it might not be possible to detect differences in adhesion strength for two bacteria of the same strain.

3.3.3 Enhanced tolerance to drying

In some food processing facilities, production is only run for part of the year, leaving part of or the whole factory idle for a longer period. This is the case in (some) fish slaughterhouses.

This inactive processing period leaves the factory dry for a long period. However, it is possible to isolate *L. monocytogenes* from such an environment left dry for nine months (Vogel et al., 2007). Similarly, *L. monocytogenes* survived in sand for 136 days at 10 °C and 0% RH (De Roin et al., 2003). One may speculate whether such drying periods might favour the survival of the persistent strains making them able to outcompete other *L. monocytogenes* strains when processing starts.

In this thesis, we found that *L. monocytogenes* survived 20 h of drying very well when protected by biological material (growth media), and survival was further increased with the addition of NaCl (Table 3.2) (Kastbjerg and Gram, 2009; Kastbjerg et al., 2009b). This difference in protective effect was not as clear after 30 min drying as after a longer drying time of 20 h. The survival was a general trait for the different strains of *L. monocytogenes* and not unique to the persistent subtypes (represented by N53-1 and V518a). When a mixed *L. monocytogenes* biofilm was grown on stainless steel and equilibrated over saturated salt solutions of 94%, 75%, 58% and 33% relative humidity for 24 h, around 10^6 CFU cm⁻² were recovered (Rodriguez et al. 2007). However, CFU cm⁻² was not determined before saturation. The survival of *L. monocytogenes* is remarkably better in media compared with desiccation in physiological peptone saline (PPS) (Vogel et al., 2007). However, ongoing work in our lab has demonstrated that *L. monocytogenes* can survive longer than ten months at 15 °C in physiological peptone saline (PPS) at low humidity. This is seen for several strains of *L. monocytogenes* and is not a unique trait for the persistent strains. Though no difference in survival, one might speculate if further studies

with desiccated strains could demonstrate an advantage for persistent strains in their growth or coping with food-stress.

Table 3.2 Number of *Listeria monocytogenes* cells on stainless steel coupons after surface application, drying for 30 min and for 20 hours at room temperature (Kastbjerg and Gram, 2009).

Strain	No	Log ₁₀ (cfu coupon ⁻¹) ± SD*					
		- NaCl			+ NaCl		
		Spotted	Dried (30 min)	Dried (20 h)	Spotted	Dried (30 min)	Dried (20 h)
N53-1	1	8.04 ± 0.06	7.87 ± 0.08	7.05 ± 0.12	8.01 ± 0.01	7.99 ± 0.05	7.84 ± 0.03 A
	2	8.32 ± 0.08	8.09 ± 0.13	6.52 ± 0.04	8.16 ± 0.01	8.16 ± 0.09	7.61 ± 0.07 A
V518a	1	8.41 ± 0.05	8.04 ± 0.12	7.00 ± 0.17	8.13 ± 0.06	8.16 ± 0.13	8.00 ± 0.09 A
	2	8.24 ± 0.01	8.28 ± 0.14	6.60 ± 0.07	8.34 ± 0.19	8.66 ± 0.04	8.01 ± 0.07 A
N22-2	1	8.16 ± 0.01	7.65 ± 0.07	6.93 ± 0.05	8.12 ± 0.10	8.07 ± 0.08	7.77 ± 0.06 A
	2	8.27 ± 0.01	8.26 ± 0.05	6.81 ± 0.13	8.15 ± 0.01	8.09 ± 0.08	7.45 ± 0.00 A
Scott A	1	8.46 ± 0.06	8.28 ± 0.05	7.44 ± 0.13	8.12 ± 0.13	8.19 ± 0.04	7.86 ± 0.04 A
	2	8.27 ± 0.01	ND [†]	7.56 ± 0.08	8.12 ± 0.08	ND	7.87 ± 0.07 A
7418	1	8.48 ± 0.04	8.45 ± 0.06	7.74 ± 0.04	8.29 ± 0	8.14 ± 0.04	7.97 ± 0.02 A
	2	8.36 ± 0.08	8.38 ± 0.05	7.64 ± 0.08	8.29 ± 0.02	8.27 ± 0.04	8.03 ± 0.19 A
4446	1	8.49 ± 0.10	8.29 ± 0.09	7.52 ± 0.15	8.39 ± 0.12	8.30 ± 0.08	8.01 ± 0.02 A
	2	8.45 ± 0.04	ND	7.47 ± 0.07	8.28 ± 0.08	ND	8.10 ± 0.03 A

*Numbers are the average of triple determinations. SD is standard deviation from the triplicate measurements

[†]For comparison of the effect of presence of 5% NaCl during growth and drying for 20 h, mean values for one strain in one trial followed by A is significantly higher (P < 0.05).

*ND, not determined

3.3.4 Enhanced survival in co-culture

In a food processing plant, a heterogeneous microbiota will be present, and the resident microbiota may have an impact on the survival and attachment of *L. monocytogenes*. Whereas *L. monocytogenes* cultured alone does not have a high potential for forming a complex biofilm, one may hypothesize that strains belonging to other genera producing a more complex biofilm structure could favour survival of *L. monocytogenes*. This cohabitation in bi- or multispecies biofilms could favour the persistent strains as compared to the presumed non-persistent strains.

Bacterial strains affect the colonization of *L. monocytogenes* in multispecies biofilms differently. *Pseudomonas fragi* (Sasahara and Zottola, 1993), *Flavobacterium* sp. (Bremer et al., 2001) and one strain of *Staphylococcus aureus* (Rieu et al., 2008b) increased the cell count of *L. monocytogenes*. Six strains of *S. aureus* had no effect on *L. monocytogenes* in binary biofilms (Rieu et al., 2008b), whereas a mixture of *Staphylococcus xylosus* and *Ps.*

fragi decreased the cell count of *L. monocytogenes* in multi-species biofilms (Norwood and Gilmour, 2001). A similar tendency was found in a larger survey with different strains isolated from a food processing environment. Sixteen strains decreased biofilm-formation, ten strains had no effect and four strains increased the *L. monocytogenes* biofilm CFU count when growing in binary biofilms. However, no link could be found between exopolysaccharide production, production capacity of the bacterial strains in pure-culture biofilms and their effect on the *L. monocytogenes* population in mixed biofilm (Carpentier and Chassaing, 2004). Instead, the different impact of strains on *L. monocytogenes* cell count could be due to growth competition and production of inhibitory substances.

So far, one study has compared adherence level of a *L. monocytogenes* type strain and a strain isolated from raw milk, denoted as persistent, and found that a mixture of *S. xylosus* and *Ps. fragi* decreased the cell count of both *L. monocytogenes* strains (Norwood and Gilmour, 2001). However, more studies are required to determine if co-existence affects persistent and presumed non-persistent strains of *L. monocytogenes* differently. Besides affecting growth, it could also protect the strains e.g. during cleaning and disinfection.

3.4 Conclusion on chapter 3

L. monocytogenes is able to reside in different food processing settings and this may increase the risk of product contamination. Specific molecular sub-types are able to persist in food-processing plants and several hypotheses have been proposed to explain the persistence trait. This include increased tolerance to food stress factors or drying, or enhanced adhesion or survival in co-culture. So far, no clear evidence has pointed out a specific trait and more studies, including more complex stress treatments, is highly relevant to elucidate this issue.

4 Chemical disinfection – effect on *Listeria monocytogenes*

Effective cleaning and disinfection procedures in the food industry are important in preventing the contamination of food-products with spoilage and pathogenic bacteria. The purpose of cleaning is to remove organic compounds present on the surfaces, whereas the aim of disinfection is to reduce micro-organisms present on food-contact surface to an acceptable level, thereby avoiding product contamination. Without proper cleaning, disinfection is useless, as remaining organic material will inactivate the disinfecting agent. Disinfectants can also be used directly on the food commodity to eliminate or control microbial growth, but this application is not discussed in this chapter. Disinfection can be performed by using chemical or physical methods. Disinfection based on chemical agents is the most widely used in the food industry. The physical methods include treatment with e.g. steam, ultraviolet light, and irradiation, and they are not considered further in this section. The present chapter describes disinfectants used in the food industry and model systems used for evaluation of disinfectant efficacy and strain sensitivity. It addresses the effect of disinfection on *L. monocytogenes*, including factors affecting the susceptibility, and evaluates the mechanisms involved in decreased sensitivity. Finally, the low prevalence of disinfectants is discussed together with the risk of disinfectants to cause cross- or co-resistance.

4.1 Disinfectants in the food-industry

There are a number of prerequisites for an efficient disinfection. Firstly, the disinfectant must have the right spectrum of activity being able to eliminate the relevant contaminants in the production site. Generally, disinfectants have a very broad spectrum of targets, since they are efficient against bacteria, viruses and fungi. However, Gram-negative bacteria tend to be less susceptible than Gram-positive bacteria (McDonnell and Russell, 1999). Secondly, it is important to use the right concentration, pH, temperature, and exposure time to obtain sufficient elimination of bacteria. Finally, cleaning of the surface prior to disinfection is necessary to remove organic compounds. Otherwise, the disinfection will be useless.

A wide range of disinfectants are available for the food-industry, and they can be divided into the following seven groups (Table 4.1) (Asselt and Giffel, 2005).

- Alcohols
- Aldehydes
- Biguanides
- (Bis)phenols
- Halogen-releasing agents (HRA)
- Peroxygens
- Quaternary ammonium compounds (QAC)

Each of the different groups include several important disinfectant agents of which the most well-known, including their applications and restrictions in use, have been summarized in table 4.1. Generally, disinfectants act on multiple targets on a specific cell, which make these compounds highly active against micro-organisms, but also potentially harmful to humans. This chapter primarily focus on their bactericidal activity. Bactericidal activity of a disinfectant is well-known, whereas the actual mechanism of action is rarely fully understood (McDonnell and Russell, 1999). Chlorine-based compounds, peroxygens, and compounds based on quaternary ammonium compounds (QAC) are the most frequently applied disinfectants in the food industry. These three groups have their advantages and disadvantages, and therefore the choice depend on the production site. Chlorine-based compounds are cheap but are easily inactivated by the presence of organic material. Furthermore, they are corrosive to metals. Peroxygens are very efficient and are inactivated to a lesser extend by organic debris. However, these compounds cannot be used on all types of material, since they are corrosive. QAC is widely used due to their adequate performance and non-corrosive nature. However, they are more expensive than chlorine (Asselt and Giffel, 2005; Holah et al., 2002; Lauridsen, 2009, Novadan, personal communication).

4.2 Testing disinfectant efficacy and strain sensitivity

Disinfectants sold for application in the food industry must have bactericidal activity in this field. The bactericidal activity can be evaluated by different standards, which in Europe are harmonized through common European standards. These describe test methods and requirements for evaluating the bactericidal activity of a disinfectant, both on planktonic (suspension test) and attached bacteria (carrier test) (Anonymous, 1997; Anonymous, 2001). In the suspension test, the disinfectant is allowed to work in high concentrations for 5 min imitating an ideal disinfection procedure. Subsequently, an aliquot is taken, neutralized, and the number of surviving bacteria is determined. The

Table 4.1. Disinfectant agents used for hygienic control in the food industry: types, application and mode of action (Asselt and Giffel, 2005; Gilbert and Moore, 2005; McDonnell and Russell, 1999; McMurry et al., 1998; Meyer, 2006)

Group	Important types	Application	Action on bacteria	Comments
Alcohol	Ethanol	Small spots	Membrane damage and rapid denaturation of proteins. Interference with metabolism. Cell lysis	Not for large industrial application due to need of high concentrations (60-90%). Flammable
	Isopropanol	Quick wipe-downs		
Aldehydes	Formaldehyde	Decontaminate rooms	Extremely reactive. Reacts un-specifically with functional groups of proteins Strong membrane association. Reacts un-specifically with functional groups of proteins. Inhibition of transport	Used less frequently in food production and processing due to toxicological considerations
	Glutaraldehyde	Instrument disinfectant		
Biguanides	Polymeric biguanides	Used in particular by the food industry	Membrane active Damage of intracellular membrane Leakage of intracellular components	Polyhexamethylene biguanides (PHMB) is a superior biocide due to lack of toxicology, colour, taste and surfactancy.
	Alexidine Chlorhexidine	Most widely applied biocide in hand-washing and oral products		
Bis-phenols	Triclosan	Soap, toothpaste, packing material, conveyer belts	Membrane-active Inhibits a specific enzyme in lipid biosynthesis	The action at a specific target increases the risk of resistance
Halogen-releasing agents	Hypochlorite	Frequently applied disinfectant in food industry	Highly oxidizing agents Irreversible change and disruption of DNA-protein synthesis.	Chlorine cheap
	Iodine		Penetrates bacteria and attacks keygroups of proteins	Iodine expensive. Staining of skin and plastic parts
Peroxygens	Peracetic acid	Frequently applied disinfectant in the food industry	Oxidation of essential cell components as lipids, proteins and DNA.	Mixture of water, hydrogen peroxide and acetic acid. Less sensitive to organic loads. Corrosive on some materials.
	Hydrogen peroxide			
Quaternary ammonium compounds		Frequently applied disinfectant in the food industry	Adsorption to cell wall Perturbation of lipid bilayer. Cytoplasmic protein aggregation. Leakage of cytoplasmic materials	More expensive than chlorine, but have residual action Non-corrosive and non-tainting monocationic agents

carrier test includes the use of planktonic cells that have been dried on a stainless steel surface at 37 °C. Disinfectant is applied to cover the film and allowed to act for 5 min. The surface is transferred to a neutralisation medium, and the number of surviving cells is quantified. The bactericidal activity is evaluated on specific strains in both tests, and the disinfectant shall demonstrate a 10^5 and 10^4 reduction of these strains for the suspension and carrier test, respectively.

The efficacy of disinfectants on planktonic and attached *L. monocytogenes* has been evaluated in several studies. Most of them have used suspension and carrier tests very like the methods described in the standards (Best et al., 1990; Briñez et al., 2006; González-Fandos et al., 2005; Grönholm et al., 1999; Jacquet and Reynaud, 1994; Van de Weyer et al., 1993; Wirtanen and Mattila-Sandholm, 1992a). Other set-ups have been developed especially for testing disinfectant effect against attached bacteria. These include simple set-ups that allow attachment of bacteria during growth (Fatemi and Frank, 1999, Gram et al., 2007; Krysinski et al., 1992; Meylheuc et al., 2006; Ren and Frank, 1993), and more sophisticated set-ups, including use of a constant-depth film fermenter (Norwood and Gilmour, 2000), a bioreactor with flow (Bremer et al., 2002) or a poloxamer-hydrogel where living cells are trapped in a gel (Wirtanen et al., 2001).

Besides evaluating disinfectant efficacy, the sensitivity of different *L. monocytogenes* strains to disinfectants has been compared to determine if differences could be found due to serotype, environmental survival, or persistence. Often, sensitivity to disinfectants is determined as a concentration measure e.g. minimal inhibitory concentration (MIC) (Aase et al., 2000; Heir et al., 2004; Lemaitre et al., 1998; Lundén et al., 2003a; Mereghetti et al., 2000; Mullapudi et al., 2008; Romanova et al., 2002; 2006; Soumet et al., 2005) or minimum concentration required to a minimum reduction in viable numbers (Aarnisalo et al., 2000; Holah et al., 2002). The MIC method is easy to perform, and many strains and disinfectants can be tested in the same experiment. However, the concentrations determined by both methods are qualitative and can overestimate the difference between strains. The set-ups used to study differences in disinfectant sensitivity of attached *L. monocytogenes* strains have primarily allowed the bacteria to attach during growth to coupons submerged in growth media (Ayebach et al., 2006; Chavant et al., 2004; Folsom and Frank, 2006; Frank and Koffi, 1990; Oh and Marshall, 1996; Ren and Frank, 1993; Stopforth et al., 2002). These methods are easy to perform, but lead to differences in the number of attached bacteria, i.e. the initial cell number before disinfection, and growth on both sides of the coupons makes horizontal disinfection non-useful. Also, spot inoculation of a specific volume of a bacterial cell suspension on a surface has been used allowing the

bacteria to attach and establish for up to four days (Aarnisalo et al., 2000; Robbins et al., 2005).

In this thesis, the aim was to compare strain sensitivity of persistent and presumed non-persistent strains of *L. monocytogenes* in a strictly quantitative manner (Kastbjerg and Gram, 2009). For this purpose, we developed two model systems that could be used to compare strain susceptibility both for planktonic and attached bacteria. The suspension test is a bactericidal test, whereas the carrier test is based on spot inoculation and drying of bacterial suspensions on coupons. In these systems, bacterial numbers were quantified after disinfection using three different concentrations of disinfectants. The purpose of this was to determine a concentration at which some bacteria actually survived the disinfection process and hence, a log reduction could be determined. This allowed calibration to a window where inter-strain comparison was possible. For both methods, the initial cell number could be standardized and quantified, and the coupons could be disinfected in a horizontal state.

4.3 Effect of disinfection against *Listeria monocytogenes*

4.3.1 Definition of resistance

Resistance is a relative term. It has primarily been used for antibiotics where, according to the microbiological definition, a strain is defined as resistant if it grows in the presence of higher concentrations of a drug as compared to phylogenetically related strains. According to the clinical definition, a strain is defined as resistant when it survives antimicrobial therapy *in vivo* (Guardabassi and Courvalin, 2006). Resistance can be established in different ways. One of the most common is to determine MIC of a given drug which is the lowest drug concentration that, under defined *in vitro* conditions, prevents the growth of bacteria within a defined period of time (EUCAST, 2000). If a strain is able to grow in a higher concentration as compared to other phylogenetically related strains, it will be defined as intermediate or resistant based on either microbiological or clinical breakpoints. Microbiological breakpoints are based on MIC distribution for a bacterial species, while clinical breakpoints are set up not only considering the MIC distribution but also *in vivo* parameters such as bacterial distribution in the host, pharmacokinetics and pharmacodynamics of the drug, and correlation of MICs with clinical outcome (Guardabassi and Courvalin, 2006). The clinical dose of antibiotics used for infection treatment is a concentration near the MIC-value. Consequently, resistance will result in treatment failures.

Similarly, the term resistance has been used for disinfectants describing situations (i) where a strain is not killed or inhibited by a concentration to which the majority of strains of that organism are susceptible, and (ii) in situations where a strain is not killed or inhibited by a concentration attained in practice. Whilst the span between MIC-values and in-use concentration of antibiotics is small, one should be aware that there is a much bigger difference for disinfectants between in-use concentration and the MIC-value. For instance the in-use concentration for QAC is 1000 µg/ml (Meyer, 2006), which is more than 60 times higher than the MIC-value detected for *L. monocytogenes* (Table 4.2). Consequently, isolates defined as resistant as per MIC-values would not normally survive the disinfection process; hence resistance would not lead to disinfection failures. Furthermore, the typical MIC-distribution for wild-type organisms covers three to four two-fold dilution steps for antibiotics (Kahlmeter and Brown, 2004). A similar distribution range has not been used in the studies of disinfection sensitivity of *L. monocytogenes* (Table 4.2). Hence, strains are defined as resistant at a low MIC. Furthermore, a resistance definition should at least be linked to a bactericidal test rather than to an inhibitory concentration, as the aim of the disinfectant is to kill the bacteria. In this thesis, I prefer to use the term less susceptible strains or “tolerant” as defined by Lewis (2007) as the ability of cells to survive killing by antimicrobials without expressing or using resistant mechanisms, describing strains with a higher MIC-value, and only isolates able to survive in-use concentrations of disinfectants as resistant.

4.3.2 Sensitivity of *Listeria monocytogenes* to disinfectants

Differences in susceptibility of *Listeria* to disinfectants have been determined in several studies (Aase et al, 2000; Best, et al., 1990; Heir et al., 2004; Holah et al., 2002; Lemaitre et al., 1998; Lunden et al., 2003a; Mereghetti et al., 2000; Romonova et al., 2006; Soumet et al., 2005; To et al., 2002). They have primarily been done with QACs since they are commonly used in the food industry. Some of the studies are large screening studies (Table 4.2). In each study, the breakpoints are set differently. However, the frequency of less susceptible strains is generally low, being less than 10%. The differences seen in MIC-values for sensitive and “resistant” strains between studies are caused by differences in methods used and in different definitions of breakpoints. Mereghetti et al. (2000) reported a correlation between serotype and decreased susceptibility to benzalkonium chloride (BC), whereas no such correlation was found in another study (Romanova et al., 2002).

Table 4.2. Studies of screening sensitivity of *Listeria* ssp. to quarternary ammonium compounds.

No. tested isolates	No. less susceptible isolates	MIC (µg/ml) “Resistant” isolates	MIC (µg/ml) Sensitive isolates	QAC Compound	Source
208	18	16	2-8	BC	Lemaître et al., 1998
200	20	4-7	< 2	BC	Aase et al., 2000
97	7	8-13	1-4	BC	Mereghetti et al., 2000
112	17	4-8	2-3	BC	Heir et al., 2004
255	108	7.5-15	1.87-5	BC	Soumet et al., 2005
114	9	16-32	4	BC	Aarestrup et al., 2007
19	5	5-6.25	0.62-1.25	BC	Romanova et al., 2002
		> 3	0.77-1.54	Clinicide	
		4	0.5-2	MCL	

BC: benzalkonium chloride, MCL: Myristalkonium chloride

Strain sensitivity of *L. monocytogenes* to other disinfectant compounds and to disinfectant products has also been studied (Aarnisalo et al., 2007; Folsom and Frank, 2006; Lourenço et al., 2009; Romanova et al., 2002; Van de Weyer et al., 1993). In general, strain differences are detected at low concentrations, whereas in-use concentrations kill all strains efficiently.

The ability of *L. monocytogenes* to persist in the food-processing environment, even though cleaning and disinfection processes are assumed to be properly done, have made several authors suggest that this could be due to decreased susceptibility or resistance to disinfectants in these specific molecular subtypes (Aase et al, 2000; Earnshaw and Lawrence, 1998; Holah et al., 2002; Heir et al., 2004; Kastbjerg and Gram, 2009; Lourenço et al., 2009). However, comparison of disinfectant sensitivity of persistent and presumed non-persistent strains of *L. monocytogenes* has not led to universally agreed conclusions. Aase et al (2002) found that 10% of *L. monocytogenes* strains isolated from food processing environments, raw products, and finished products had increased tolerance to BC (Table 4.2). Strains with enhanced BC tolerance that were isolated from a Norwegian fish processing plant were all persistent, indicating that such increased tolerance could be a factor correlated to persistence. However, other studies have not been able to demonstrate a correlation between persistence and enhanced tolerance to disinfectants (Earnshaw and Lawrence 1998; Holah et al., 2002; Heir et al., 2004; Kastbjerg and Gram, 2009; Lourenço et al., 2009). These studies were primarily performed with planktonic bacteria. One may speculate that a different pattern of sensitivity tolerance would be revealed by targeting attached bacteria, since sensitivity of a strain depends on the test method used. Strains regarded as less sensitive determined with MIC were more or as sensitive as other stains in

suspension or surface tests (Aarnisalo et al., 2007), and no association were found between attached chlorine tolerant bacterial cells and planktonic chlorine tolerant bacterial cells (Folsom and Frank, 2006). We studied the sensitivity of persistent and presumed non-persistent strains to two commonly used disinfectant products for both planktonic and attached bacteria (Kastbjerg and Gram, 2009).

Planktonic bacteria were screened with three different concentrations of the two disinfectants, Incimaxx DES, with peroxy acids and hydrogen peroxide as active ingredients, and Triquart SUPER with QAC. Disinfecting with 0.0031% Incimaxx DES caused 2-5 log₁₀ reduction and allowed inter-strain comparison of sensitivity differences. Strain differences were seen (Fig 4.1). However, the persistent RAPD types were as susceptible to Incimaxx DES as planktonic bacteria as compared with remaining strains. A concentration of 0.02% Triquart SUPER allowed inter-strain comparison of *L. monocytogenes* strains, and the persistent strains were as susceptible to this disinfectant as compared to other presumed non-persistent strains of *L. monocytogenes*.

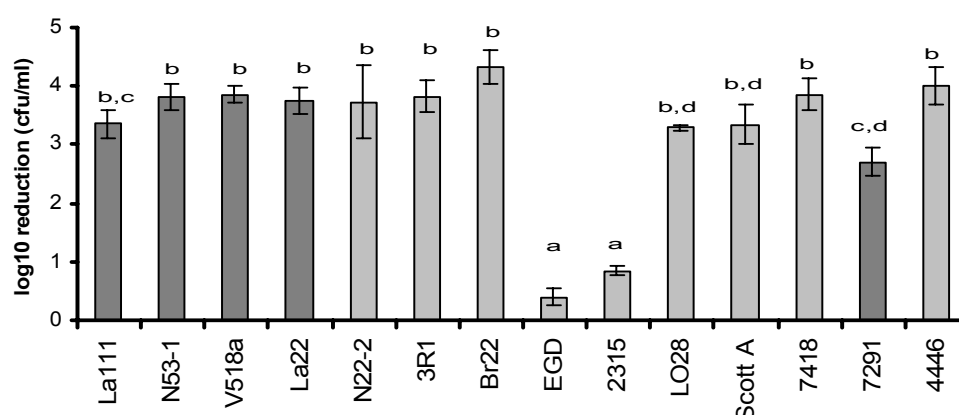


Fig. 4.1. Reduction in cell-number of *L. monocytogenes* strains after 20 min disinfection with 0.0031% Incimaxx DES. Persistent strains are in dark grey and other presumed non-persistent strains in light grey. Columns are average from one trial carried out in triplicate. Error bars are based on the standard deviation from the triplicate measurements. The results are representative of two independent experiments. For comparison of the differences in reduction between strains, columns that are not denoted by the same letter (a, b, c, d) are significantly different ($P < 0.05$) (Kastbjerg and Gram, 2009)

Similar results were seen for spot inoculated and dried *L. monocytogenes* cells (Fig. 4.2). Disinfection with 0.062% Incimaxx DES and 0.08% Triquart SUPER allowed inter-strain comparisons and minor differences in sensitivity were found between strains. However, the

persistent RAPD types, represented by N53-1 and V518a, were not more tolerant than the other strains. Hence, it seems unlikely that the ability of specific molecular sub-types of *L. monocytogenes* to persist is due to increased tolerance or resistance to disinfectants.

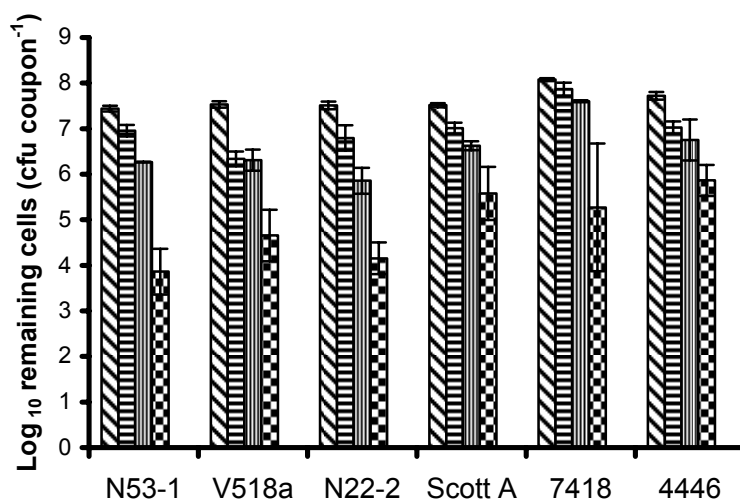


Fig 4.2. Remaining cell-number of *L. monocytogenes* strains on stainless steel after 20 h drying (▨) and 20 min treatment with water (▤), 0.031% (▥), 0.062% (▧) and 0.125% (▩) Incimaxx DES. Persistent strains are N53-1 and V518a. Columns are average from one trial carried out in triplicate. Error bars are based on the standard deviation from the triplicate measurements. The results are representative of two independent experiments (Kastbjerg and Gram, 2009).

L. monocytogenes is generally sensitive to disinfectants and can be eliminated with concentrations lower than in-use concentrations in model systems. For instance, a concentration of 0.125% eliminated spot inoculated and dried *L. monocytogenes*, which is eight times lower than the recommended minimum in-use concentration of 1% (Kastbjerg and Gram, 2009). However, it is possible to isolate both persistent and sporadically isolates of *L. monocytogenes* after the disinfection process (Vogel et al., 2007), and one may speculate why they are not eliminated during cleaning and disinfection. One reason could be that the bacteria are not exposed to the right concentration of disinfectant during the cleaning and disinfection process. This could be due to less efficient cleaning; leaving organic material on surfaces that consequently inactivates the disinfectant. Also, a poor equipment design can prevent the cleaning materials and disinfectants to reach all niches. Finally, an unequally distribution of the disinfectant in the processing plant, and a poor knowledge on important contaminations sites as floor and drains also reduce the ability to eliminate *L. monocytogenes* sufficiently.

4.3.3 Influence of pre-growth condition on disinfection sensitivity of *L. monocytogenes*

It is well-known that the presence of organic material affects disinfection efficiency making the disinfection procedure less efficient both for planktonic and attached cells (Aarnisalo et al., 2000; Best et al., 1990; Van de Weyer et al., 1993). Some disinfectants are more affected by the presence of organic material than others, and the type of soiling influences the efficiency of the disinfectants differently (Best et al., 1990; Gram et al., 2007; Wirtanen and Mattila-Sandholm, 1992b). Also, pre-growth conditions of *L. monocytogenes* can influence the efficiency of the disinfectant.

Growth temperature affected the inactivation of *L. monocytogenes* cells, as bacterial cells grown at 6 °C and 21 °C were more susceptible than bacterial cells grown at 35 °C when exposed to chlorine (Lee and Frank, 1991). However, the difference was most pronounced at short exposure times of 30 s. No difference was found between exponential and stationary planktonic bacteria in sensitivity to QAC (Chavant et al., 2004). However, this depends on concentration, since Luppens et al. (2001) found similar reduction in viable cell number for stationary-phase bacteria and exponential-phase bacteria at a one concentration, whereas exponential bacteria were more susceptible than stationary-phase bacteria at a higher concentration of QAC. Duration of the incubation time affected the sensitivity of attached bacteria to QAC. Sensitivity to QAC decreased with incubation up to 7 days (Chavant et al., 2004), whereas attached bacteria were more sensitive to chlorine with increasing age (up to 7 days) (Wirtanen and Mattila-Sandholm, 1992b). Stopforth et al. (2002) found that *L. monocytogenes* displayed stronger tolerance to the effects of three sanitizers including chlorine, QAC and PAA on day 7 than on day 2, but had become sensitized to all sanitizers by day 14. Similarly, 7-day attached cells of *L. monocytogenes* were less inactivated, when exposed to monolaurin and acetic acid, than with younger *L. monocytogenes* cells (Oh and Marshall, 1996). Conflicting results can be due to differences in methods, compounds, and concentrations used. Starvation for four days in PBS slightly reduced sensitivity of planktonic bacteria to QAC, whereas it had no effect on the susceptibility of attached *L. monocytogenes* (Ren and Frank, 1993). No difference between previously acid-adapted and non-adapted *L. monocytogenes* was detected with regard to sensitivity to disinfectants (Stopforth et al., 2002).

In this thesis, we found that pre-growth with 5% NaCl increased the tolerance of planktonic *L. monocytogenes* cells to Incimaxx DES, but not to Triquart SUPER (Kastbjerg and Gram, 2009). This was a general trait and not only observed for the persistent *L. monocytogenes* sub-types. However, the growth and spot inoculation with 5% NaCl, did not protect the attached *L. monocytogenes* further during disinfection as compared to

growth and spot inoculation without NaCl. In contrast, cultivation in diluted TSB (1:15) increased tolerance of *L. monocytogenes* to QAC (Lee and Frank, 1991). A minor increase was due to the decrease in NaCl which was further increased in the combination with dipotassium phosphate (Ren and Frank, 1993). Hence, it is not clear if differences in pre-growth could affect sensitivity of strains to disinfection procedures in the industry.

The influence of pre-growth has been determined when disinfecting with concentrations lower than in-use concentrations. However, the bacteria might be exposed to sub-lethal concentrations in the industry and hence, environmental parameters of relevance for a particular food production should be included when studying the inactivation of pathogenic bacteria. However, so far it seems unlikely that pre-growth differences could explain the ability of specific molecular sub-types of *L. monocytogenes* to persist, since they were found equally sensitive to food stresses as discussed in chapter 3.

4.3.4 Disinfection sensitivity of planktonic versus attached *L. monocytogenes*

Generally, a higher concentration of disinfectant is needed to eliminate attached bacteria as compared to planktonic bacterial cells, including *L. monocytogenes* (Folsom and Frank, 2006; Frank and Koffi, 1990; Norwood and Gilmour, 2000; Robbins et al., 2005; Stopforth et al., 2002). However, in the cited studies, different set ups have been used for the disinfection of planktonic and attached cells, respectively, leading to differences in initial cell number and presence of biological material. As biomass and presence of biological material influence the efficiency of disinfectants, it cannot be concluded if the higher concentration required to eliminating attached bacteria, as compared to suspended bacteria, is due to a higher biological load introduced by the set-up or to increased tolerance of the attached bacteria *per se*. In this thesis, when the two model systems used were calibrated to include equal organic material (substrate and bacteria), and equal volumes and concentrations of disinfectants, *L. monocytogenes* was equally sensitive to disinfectants whether attached or suspended (Fig. 4.3) (Kastbjerg and Gram, 2009). Interestingly, Frank and Koffi (1990), and Stopforth et al. (2002) both reported that a higher concentration was needed for the elimination of attached bacteria, but the susceptibility of detached *L. monocytogenes* cells to disinfectants was equal to that of cells in suspension. Together, this indicates that the higher tolerance of attached bacteria to disinfectants is due to one or more of the following factors: (i) protection from organic material, (ii) decreased possibility for the disinfectant to penetrate the cell or (iii) cell-changes only present when the cell is attached.

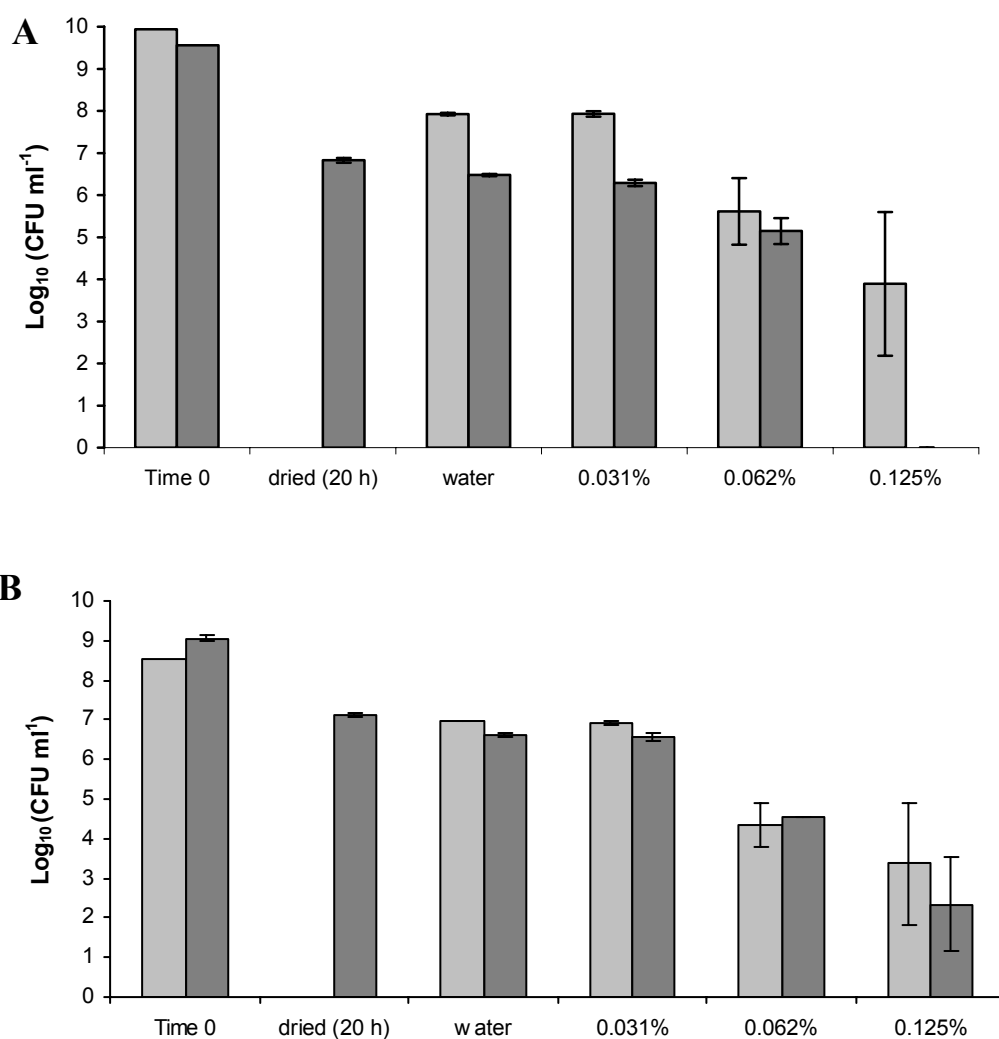


Fig. 4.3. Number of *L. monocytogenes* N53-1 bacterial cells after 20 min disinfection of planktonic (light gray) and attached (dark grey) cells with Incimaxx DES. The experiments were performed with planktonic cells re-suspended in 10-fold less volume (a) and planktonic cells re-suspended in the same volume (b). The attached cells were up-concentrated in both experiments. Columns are average of triplicate determinations. Error bars are based on standard deviations from the triplicate measurements (Kastbjerg and Gram, 2009).

4.4 Mechanisms of decreased susceptibility to disinfectants

The observed difference in sensitivity between strains of *L. monocytogenes*, and the possibility to adapt sensitive strains of *L. monocytogenes* to an increased MIC (Aase et al., 2000; To et al., 2002) have led to further investigations of the mechanisms causing susceptibility changes. The decreased susceptibility was caused by different mechanisms, dependent on the original sensitivity of the *L. monocytogenes* strain. In adapted strains (i.e. originally sensitive strains with increased MIC caused by a repeated exposure to sub-lethal concentrations of disinfectants in the laboratory), the decreased susceptibility of *L. monocytogenes* to QAC was at least partially caused by reduced intracellular accumulation due to activation of efflux pumps (Aase et al., 2000; Romanova et al., 2006; To et al., 2002). All isolates of *L. monocytogenes*, independent of QAC sensitivity, carried the *mdrL* gene, which encodes a multidrug efflux pump, and the *orfA* gene, a putative transcriptional repressor of *mdrL* (Mereghetti et al., 2000; Romanova et al., 2002). This could indicate that decreased susceptibility to QAC not was due to the *mdrL* and *orfA* genes, since they were ubiquitous. However, adaptation to BC of sensitive *L. monocytogenes* isolates in the laboratory resulted in a significant increase in expression of *mdrL*, whereas low and intermediate expression levels were detected in originally tolerant strains (Romanova et al., 2006). Hence, the originally tolerant strains may have an increased MIC due to another mechanism than the *mdrL* efflux pump. This could be due to changes in the cell wall, since *L. monocytogenes* strains with originally decreased susceptibility to QAC were found to have modifications in their cell wall (Mereghetti et al., 2000; To et al., 2002). The same difference in mechanisms due to the original sensitivity of the strain has been observed in staphylococci (Heir et al., 1999).

There is no clear indication if the decreased susceptibility of some *L. monocytogenes* strains to disinfectants is due to the presence of plasmids. Mereghetti et al. (2000) and Soumet et al. (2005) found that less susceptible strains of *L. monocytogenes* conserved their tolerance to QAC after plasmid curing, whereas others have found a connection between increased tolerance and the presence of plasmids (Lemâitre et al., 1998; Romanova et al., 2002). These conflicting conclusions can be caused by the poor knowledge of the mechanisms involved. As the disinfectants attack several targets on the cell, several resistance mechanisms may play a role. Hence, there may be more than one efflux pump or mechanism acting, which can be chromosomally or plasmid bound. Furthermore, the strains studied only have a limited increase in MIC, indicating that true resistant mechanisms might not be involved.

4.5 Differences between antibiotics and disinfectants

The increased selective pressure imposed by the widespread use of antibiotics since 1950s has distinctly accelerated the development and the spread of bacterial resistance to antibiotics. In most cases it took no longer than 3 to 5 years after introduction of a broad-spectrum antibiotic into clinical use, until the first resistant target bacteria occurred (Schwartz and Chaslus-Dancla, 2001). In contrast, many disinfectants have retained their effect during more than 100 years of use (Hugo, 1991), and despite the widespread use of disinfectants in the food industry, data on resistance to disinfectants in *L. monocytogenes* and other food-related bacteria (Heir et al., 1995; Sindhu et al., 2001; 2002; Sundheim et al., 1992) isolated in the plant or in the finished product are scarce. This low prevalence of resistance to disinfectants among food-related bacteria is likely due to differences between antibiotics and disinfectants with respect to target and use.

An ideal antibiotic is different from an ideal disinfectant with respect to the targets. An ideal antibiotic possesses a single biochemical target that is absent from the host, whilst disinfectants generally possess a number of distinct targets, with varied susceptibilities, that may also occur in eukaryotic systems (Table 4.2) (Gilbert et al., 2002). Due to the multi-target nature of the disinfectants, mutations within a single target are unlikely to result in treatment failure. One exception is resistance to triclosan which is related to mutations in the *fabI* gene encoding the enoyl-acyl carrier protein reductase of fatty acid biosynthesis (McMurry et al., 1998), even though, other studies find additional cellular targets for triclosan (Russell 2003). This is still the only example of a disinfectant with a specific target. Hence, resistance to disinfectants might require changes in structural components of the bacterium that physiologically are too damaging for it to survive.

As antibiotics are used in treatment of a host, disinfectants in processing areas are used on inert materials. Antibiotics are used in concentrations near the MIC-value, since the aim is to inhibit the growth of the pathogenic bacteria in combination with the immune system of the host and to protect the beneficial microflora in the host. At these concentrations it may be possible for bacteria to overcome the treatment and develop resistance. In contrast, disinfectants are in general used at very high concentrations relative to their MIC (if applied appropriate). At these levels, it is impossible for bacteria to overcome the rapid massive damage and develop resistance. If the bacteria are exposed to sub-lethal disinfectant concentrations and survive, the development of resistance is more difficult as discussed above. A maximum limit exists for increasing the dose of antibiotics further due to the host. On the other hand, there is theoretically no limit for disinfectants, away from cost and environment, as long as it is used for equipment.

The application of disinfectants and antibiotics are different. While disinfectants are only used for eliminating bacteria, some antibiotics are still used preventively in different ways. In animal flocks, antibiotics can be used for prophylaxis, as therapy for sick animals combined with prophylaxis in healthy (metaphylaxis), and for growth promotion, using the antibiotic at sub-therapeutic concentrations leading to increase in growth (Guardabassi and Courvalin, 2006). This may select for resistant bacteria that can be spread to humans through food products like meat and eggs. Furthermore, these resistant bacteria constitute a reservoir of resistance genes that may transfer to and cause problems for humans. Even though use of antibiotics as growth promoters has been totally banned in countries of the European Union from 2006, they are still used intensively in other parts of the world, including the US and Australia (Guardabassi and Courvalin, 2006). This difference in the way of using the compounds can influence the difference of resistance development. One could argue, in relation to what have been mentioned before, that if the bacterium is exposed to disinfectant levels lower than those required to kill the organism it may lead to adaptation and generation of decreased susceptibility as well. However, there will be differences in the concentration used. For antibiotics, the preventive dose will be subtherapeutic and constant. For disinfectants, the concentration is typically higher than the MIC-value and it will probably not be constant with steady-state increases from day to day as used in laboratory in adaptation studies.

4.6 Cross- and Co-resistance to antibiotics

So far, a low prevalence of resistance to disinfectants is found in *L. monocytogenes* and in other food-related bacteria (Heir et al., 1995; Sindhu et al., 2001; 2002; Sundheim et al., 1992). However, concern has been expressed that exposure to sub-lethal concentrations of disinfectants may lead to development of resistance mechanisms to components that attack the cells more specifically as e.g. antibiotics. This is referred to as cross- or co-resistance dependent on the mechanism involved (Chapman, 2003).

Application of disinfectants in sub-lethal concentrations can affect antibiotic susceptibility in laboratory models. Sub-lethal concentrations of QAC showed the highest selectivity for variants of *Salmonella* Typhimurium with reduced susceptibility to different antibiotics (Karatzas et al., 2007). Some of these variants had increased level of *acrB*, a marker for efflux leading to increased sensitivity to antibiotics. Also, induction of efflux pumps caused by low-level of biocides (antiseptics, disinfectants, preservatives) has been detected in other pathogens such as clinical *S. aureus*, *Stenotrophomonas maltophilia* and

Pseudomonas aeruginosa (Fraud et al., 2008; Huet et al., 2008; Sanchez et al., 2005). All studies were done with adapted isolates.

In *L. monocytogenes*, decreased susceptibility to BC has been linked to resistance to ethidium bromide (EB). However, it was not a general trait for the BC tolerant strains, since only part of the strains, 14 of 18 (Lemaître et al., 1998) and 10 of 20 (Aase et al., 2000) were resistant to EB as well. The EB resistance was based on a proton motive force driven efflux pump (Aase et al., 2000). There was no indication that decreased susceptibility to BC was related to resistance to 15 different antibiotics tested. Similarly, others did not find any cross-resistance in *L. monocytogenes* with QAC and antibiotics (Aarestrup et al., 2007; Soumet et al., 2005). However, adaptation of *L. monocytogenes* by serially sub-culturing in BC led to a two- to fourfold increase of MICs to the antibiotics gentamicin and kanamycin, and also to two other QACs, whereas resistance to other types of disinfectants or antibiotics did not change (Romanova et al., 2006). In contrast, Lunden et al. (2003a) observed cross-adaptation after exposure to increasing concentrations of a disinfectant both for related and unrelated disinfectants. Though, the MIC of disinfectants due to cross-adaptive responses was similar to or smaller than the MICs of disinfectants resulting from adaptive responses. So far, cross-resistance to antibiotics seems to be minimal in naturally occurring *L. monocytogenes*, whereas adaptation with sub-lethal concentrations led to increase in MIC to some antibiotics.

Resistance to disinfectants and cross-resistance to antibiotics can hypothetically develop, as this can be obtained under *in vitro* conditions in the laboratory. However, so far there is no evidence that this is a major problem for *L. monocytogenes* and other food-related bacteria in the food industry. It is, however, important to be aware of disinfectants that act more specifically, like triclosan, since resistance mechanisms to such products may evolve more easily. Furthermore, the tendency to incorporate disinfectant compounds in materials to obtain an anti-bacterial effect might cause a higher resistance pressure, which might increase antibiotic resistance. Hence, it is important to follow the resistance development to disinfectants and to understand how disinfectants in sub-lethal concentrations affect bacteria to ensure that such problems will not accelerate.

4.7 Conclusions on chapter 4

The disinfection process is important in the food industry in order to control the contamination of food-products with spoilage and pathogenic bacteria. Hence, resistance or increased tolerance to disinfectants used could explain the ability of specific sub-types of *L. monocytogenes* to persist in the food processing environment. However, so far there is no

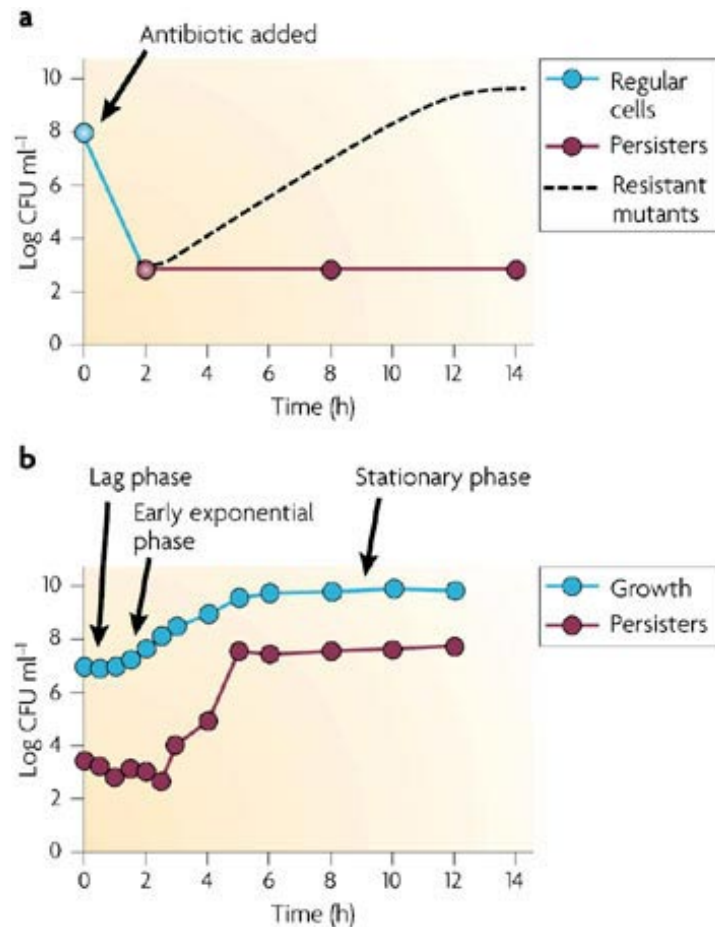
indication that persistent sub-types of *L. monocytogenes* are less susceptible to disinfectants used in the food-industry as compared to presumed non-persistent strains.

5 Stress on single cell level in *Listeria monocytogenes*

Bacteria often behave as a population and several phenotypes are regulated in a cell density dependent manner. However, they are foremost single-cell organisms that under natural conditions constantly cope with alterations in the environment. These are counteracted by a stress response that typically involves the activation of specific sets of genes in response to particular environmental conditions. The often unstated assumption in considering such phenomena is that cells respond in a more-or-less uniform manner to the inducing signals, because the techniques classically used in these studies determine average values across an entire population. However, concurrently with the ability of single-cell studies, several examples have emerged of non-uniform physiological response in clonal populations of bacteria referred to as bistability (Dubnau and Losick, 2006), phenotypic variation (Smits et al., 2006) or phenotypic heterogeneity (Avery, 2006). The different terms refer to the same phenomenon: presence of individual cells in an isogenic population that exhibit a dissimilar phenotype.

5.1 Phenotypic heterogeneity

More than 60 years ago, Bigger observed that a small number of *S. aureus* cells survived when challenged with penicillin and these cells resumed growth when the antibiotic was removed (Fig. 5.1a) (Bigger, 1944). When retreated with penicillin, the bacterial population were found to be just as sensitive as the original cells but once again a small number of cells survived a second round of drug challenge. Hence, the surviving fraction was not antibiotic resistant in the traditional genetic sense. This surviving cell fraction was denoted as persister cells, defining bacteria able to survive an antibiotic treatment and to resume growth after removal of the drug. A similar presence of persister cells has been observed in other bacteria exposed to a wide range of antibiotics, and also in biofilms (Handwerger and Tomasz, 1985; Keren et al., 2004a; Rinder et al., 2001; Spoering and Lewis, 2001; Sufya et al., 2003; Wiuff et al., 2005). The population of persister cells increases in the later state of the exponential phase and stationary phase (Fig 5.1b), and they are preformed rather than being produced in response to antibiotics (Balaban et al., 2004; Keren et al., 2004a).



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Fig. 5.1. Formation of persister cells. (A) Treatment of a population with antibiotic results in cell death, leaving only persister cells or resistant mutants alive. (B) The frequency of isolation of persisters as a function of the growth phase of the culture (copied from Lewis, 2007).

The presence of persister cells during exposure to antibiotics is clinically relevant as they might be important in the many recalcitrant infectious diseases. However, little is known about persister cells due to technical difficulties in the isolation of, and working with, a small fraction of cells, typically 10^{-6} to 10^{-4} (Keren et al., 2004a). However, the isolation of “high persistence” types of *E. coli* that produced more persister cells than the wild-type strain (Moyed and Bertrand, 1983) has led to insight into the nature of the persistent state. This has indicated that a small number of cells in the population spontaneously enter and later exit a state of no or slow growth where they can survive drug treatment (Balaban et al., 2004). Several toxin-antitoxin (TA) modules were up-regulated in persisters (Keren et al., 2004b). Typically, the toxin inhibits important cellular functions such as translation or replication and forms an inactive complex with the antitoxin. The toxin is stable whereas the antitoxin is degradable. The observation that persisters are isogenic cells with altered

genetic expression compared to “normal” cells, and that a given cell can switch back and forth between growth and non-growth has lead to the proposal that the persister cells themselves are in a dormant state and hence, the observed down-regulation of biosynthetic pathways and slow growth protects them from antibiotic attack (Lewis, 2007). However, this may not be the case in all situations since fluoroquinolones that are capable of killing non-growing cells are nonetheless incapable of eliminating persisters (Keren et al., 2004b; Dhar and Mckinney, 2007). Others have argued that persister cells are generated due to asymmetric ageing and hence, their changed phenotype can be explained by senescence (Klapper et al., 2007; Stewart et al., 2005).

Phenotypic heterogeneity has been observed during conditions other than antibiotic exposure. Survival differences in a population were also observed during exposure to other stresses. When an exponential culture of *Escherichia coli* was exposed to a rapid transfer to pH 3, a small fraction remained culturable for several hours. When outgrown at pH 7, the survivors generated a population of cells that was almost identical in acid sensitivity to the culture from which it was selected (Booth et al., 2002). Similarly, outgrowth of a population of survivors challenged to osmotic shifts yielded a population that split into a proportion of non-survivors and survivors in subsequent survival assays (Booth et al., 2002; Levina et al., 1999). Another example is the natural occurrence of competence in *Bacillus subtilis*, where the DNA transport and recombination proteins are synthesized in only 10% of the cells. These cells are not genetically distinct, and when competent or non-competent cells from the same culture are returned to initial conditions, again 10% express competence in stationary phase (Dubnau and Losick, 2006). Similar association of competence with sub-populations of a genetically homogenous bacterial culture has been observed in other strains (reviewed by Smits et al., 2005). Phenotypic heterogeneity is also observed during spore formation in *B. subtilis*, which is activated under conditions where cells are limited for nutrients. Some cells activate the key sporulation regulator Spo0A and some does not, leading to a mixture of sporulating and non-sporulating cells (Chung et al., 1994).

5.2 Methods to detect and study sub-populations

The presence of sub-populations displaying a heterogenic phenotype from the principal bacterial population can be determined in several ways. Counting colony forming units is useful and still valuable to determine small fractions of survivors as persister cells. However, it requires growth, and it is only useful to determine survival heterogeneity whereas other sorts of phenotypic heterogeneity at single cell level are masked. Flow cytometry is a rapid method usable for measurements at the single cell resolution

(reviewed by Davey and Kell, 1996), and it has been used for several purposes including detection of differences in gene expression (Chung et al., 1995), respiratory activity (Kaprelyants and Kell, 1993), and cell division (Roostalu et al., 2008). It enables large numbers of cells to be analyzed rapidly and the population's response to changes in environment to be monitored. However, it cannot follow the same single cells over time and cannot be used for attached bacteria. Microscopy can be used to study single cells over time, both in liquid culture and as attached cells. Though, a disadvantage of the microscope methods is the requirement of a more than 10^6 cfu/ml in the suspension due to the detection limit. One method is optical microscopy combined with microfluidic devices following growth of single cells (Balaban et al., 2004). This has been used to study differences in growth rate of *E. coli* cells exposed to an antibiotic. Another technique for *in situ* analyses of growth and other physiological conditions of single cells is fluorescence ratio imaging microscopy (FRIM) based on fluorescence microscopy and ratio imaging of bacterial cells (Siegumfeldt et al., 1999).

FRIM enables studies of dynamic changes with high sensitivity and on single cells with the ability to follow the same single cells over time. It has been developed for measuring intracellular pH (pHi) in single bacterial cells as indicator of stress and viability (see below), and it combines use of a pH dependent fluorescent probe in conjugation with a microscope and an image analysis system (Siegumfeldt et al., 1999). A very suitable probe is carboxyfluorescein, as it binds covalently to primary amines in the cytosol and hence, is not lost from the cell under leakage (Fig. 5.2a) (Breeuwer et al., 1996). However, the pH range of the probe is limited from pHi 5 to pHi 9. The fluorescence intensity is dependent on both probe concentration and pH. Consequently, to obtain a pHi-value that is independent of probe concentration, the bacterial cells are excited at two different wavelengths with emission dependent and independent of pH, respectively (Fig. 5.2b). By dividing the two emission values, the ratio value only reflects the pHi. The use of different chambers with or without flow gives the ability to control the cell environment during analysis. FRIM has been used to study changes in pHi in single cells of bacteria during co-culture, osmotic and acid stress, and exposure to different bacteriocins (Budde and Jakobsen, 2000; Fang et al., 2004; Hornbæk et al., 2006; Shabala et al., 2002; Siegumfeldt et al., 1999).

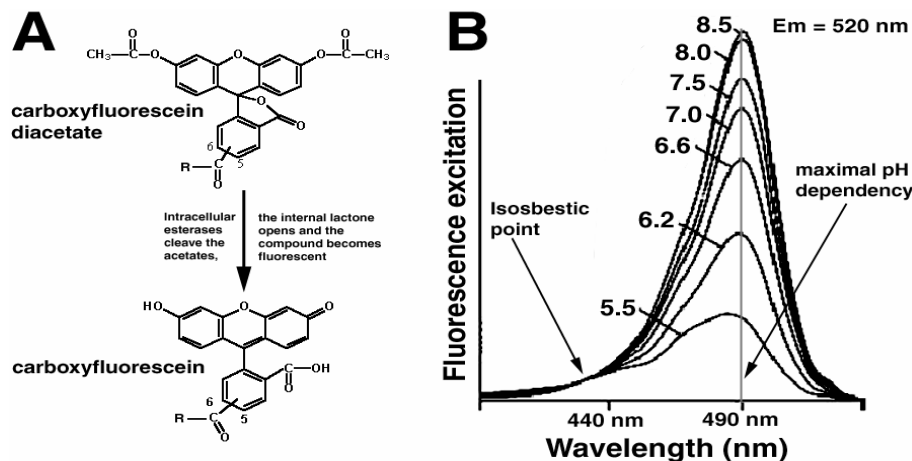


Fig 5.2. (A) The non-fluorescent precursor carboxyfluorescein diacetate is hydrolysed by intracellular esterases, and the resulting carboxyfluorescein is highly fluorescent. (B) A schematic excitation spectrum of a fluorescein derivative, showing the isosbestic point, which enables the acquisition of a concentrations independent ratio $R_{488nm/435nm}$ (Copied from Siegmundfeldt, 2000).

5.3 Response of *Listeria monocytogenes* at single cell level to stresses

Homeostasis refers to the ability of a living organism to maintain an approximately constant internal environment despite changes in the external surroundings. This includes maintenance of an pH_i in an appropriate interval for the bacteria to ensure the metabolic reactions to occur and thereby maintain viability. Bacteria can be divided into neutrophiles, acidophiles and alkalophiles dependent on their pH optimum for growth, and it is recognized that each group exhibit pH_i values in a specific range (Booth, 1985). Hence, during optimum growth conditions, acidophiles exhibit pH_i in the range of pH_i 6.5 to 7.0, neutrophiles pH_i 7.5 to 8.0, alkalophiles 8.4 to 9. *Listeria* belongs to the group of neutrophiles and maintains its pH_i within a narrow range of 7.6-8 at extracellular pH (pH_{ex}) values of 5.0 to 8.0 (Budde and Jakobsen, 2000; Siegmundfeldt et al., 1999), and at pH_{ex} of 4.0 with the presence of glucose (Shabala et al., 2002). It is believed that viable cells need to maintain a transmembrane pH with their pH_i above the pH_{ex} , and failure to maintain pH_i indicates that the bacterial cell is stressed and leads to loss of cell viability (Budde and Jakobsen, 2000; Chitarra et al., 2000; Hutkins and Nannen, 1993; Shabala et al., 2002).

A homogenous but significant decrease was observed in pH_i in *L. monocytogenes* during exposure to osmotic stress, and it was more pronounced when grown on agar substrate as in liquid culture (Fang et al., 2004). However, the bacterial cell was able to recover over time during exposure of up to 15% NaCl. Acid stress also caused a homogenous decrease in pH_i in *L. monocytogenes*, but the decrease was counteracted by the addition of glucose (Shabala et al., 2002). The trend in pH_i changes was similar to the trend in survival.

Exposure of *L. monocytogenes* to pHex 3.0 for two hours revealed a pH_i of ≤ 5 . However, the strain remained viable as it recovered immediately and remained constant at pH_i 7.3, when returning to pHex 6.0.

Interestingly, exposure of *L. monocytogenes* to different bacteriocins caused a non-homogenous decrease of pH_i. Differences in nisin sensitivity was observed for *L. monocytogenes* cells grown on a agar medium under similar conditions (Budde and Jakobsen, 2000), and treatment with sub-inhibitory concentrations of leucocin and nisin gave rise to two sub-populations; one consisting of cells with a dissipated pH gradient (Δ pH) and the other consisting cells that maintained Δ pH (Fig. 5.3) (Hornbæk et al., 2006). This occurrence of sub-populations is more likely due to phenotypic heterogeneity than to bacteriocin resistance as the Δ pH was dissipated for all single cells at high concentrations of bacteriocin.

Hence, one may speculate how treatment with a sub-lethal concentration of disinfectant affects the bacterial population of a persistent strain of *L. monocytogenes* and how it attacks the population - is only a fraction of the cell population attacked, leaving another fraction of cells unaffected. In case of the latter, some bacterial cells may be able to survive the disinfection treatment, which could, ultimately, ensure that the genome is propagated leading to persistence. Others have followed pH_i over time for exponential and stationary-phase cells of *L. monocytogenes* during exposure to benzalkonium chloride (Luppens et al., 2001). However, the pH_i was measured on a population basis using spectrophotometry and single cells could not be followed. Hence, we exposed a persistent sub-type of *L. monocytogenes* to Incimaxx DES, a peroxygen disinfectant, in three different concentrations and studied the effect on single cell level by measuring pH_i and also compared to viability (Kastbjerg et al., 2009b).

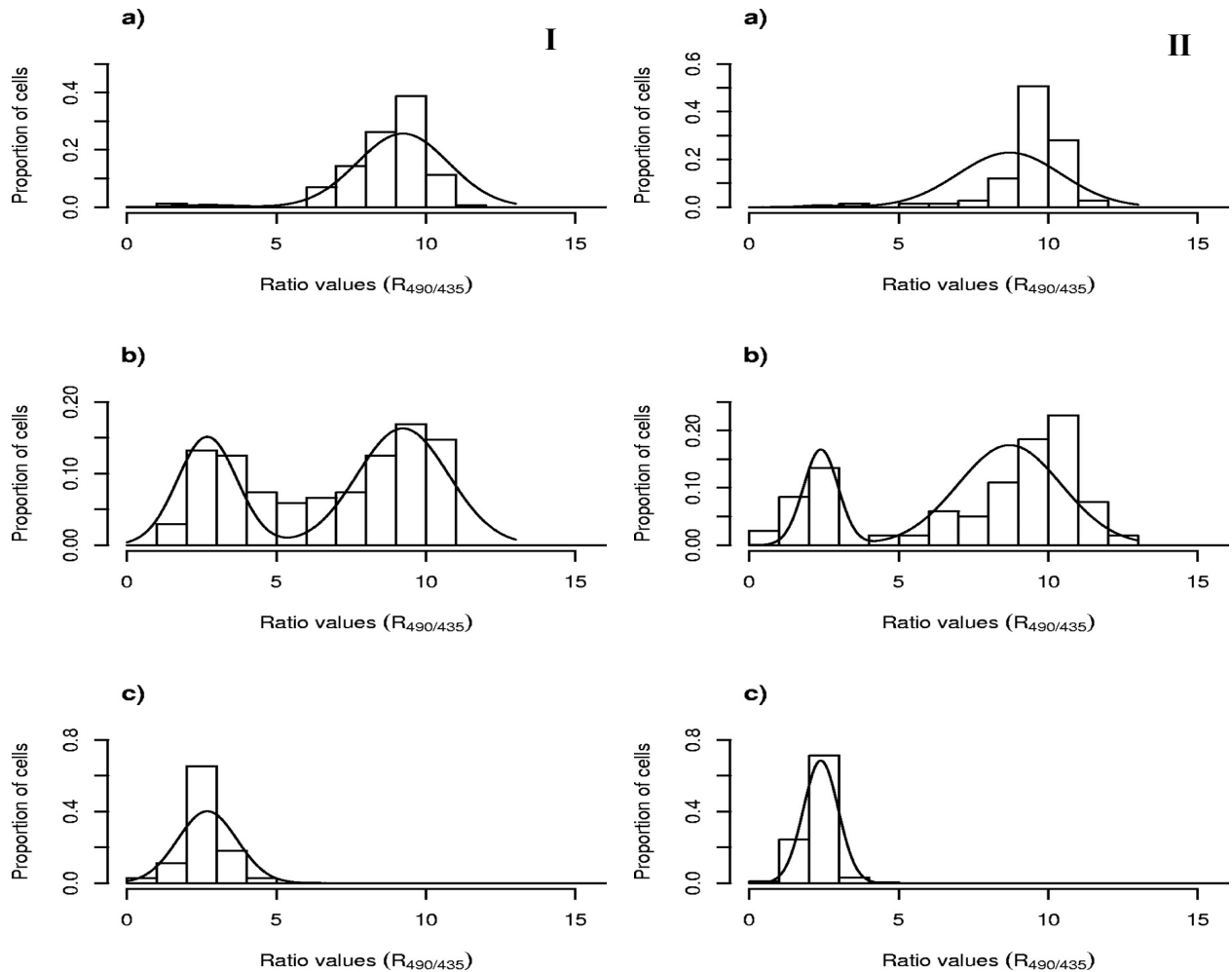
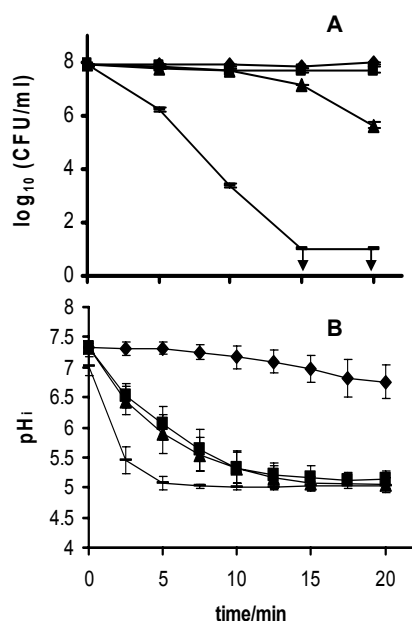


Fig. 5.3. Distributions of R490/435 values of single cells of *Listeria monocytogenes* 4140 cells immobilized on a filter membrane and measured by FRIM upon exposure to (I) leucocin 4010 at concentrations of 0 (a), 9,600 (b), and 24,000 (c) AU/ml or (II) nisin at concentrations of 0 (a), 400 (b) and 1,800 (c) IU/ml (copied from Hornbæk et al., 2006).

pHi decreased during the 20 min treatment to pHi 5 for all three concentrations of Incimaxx DES, indicating that the bacterial cells were stressed (Fig. 5.4b). However, the rapidity were dependent on disinfectant concentration as pHi decreased to less than 5.5 in 5 min of treatment with 0.0062% Incimaxx DES, whereas the change was slower for 0.0031% and 0.0015% Incimaxx DES. Demineralized water caused only a minor decrease from pHi 7.33 ± 0.06 to pHi 6.75 ± 0.28 during the 20 min treatment. The decrease in pHi during disinfection is due to the disinfectant and not an effect of low external pH, as the extracellular pH in the bacteria-disinfection solutions was around 4.0 with all three concentrations of Incimaxx DES including water. The change in pHi was similar in all cells measured, indicating that the response of the bacterial population was homogenous. Similarly, for spot inoculated and dried bacterial cells, disinfection with Incimaxx DES caused a homogenous decrease in pHi. Hence, no sub-populations were detected.

I



II

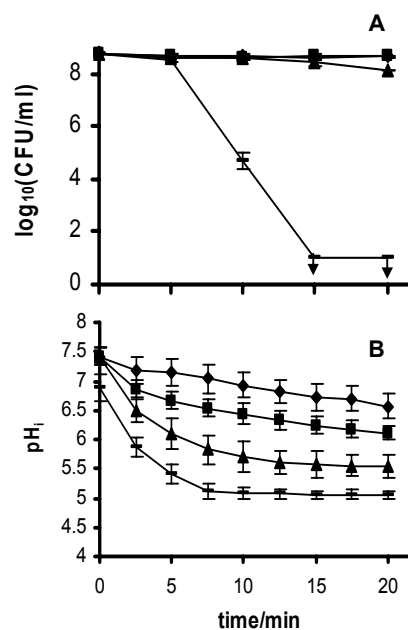


Fig. 5.4. Viable counts (A) and pH_i (B) of *Listeria monocytogenes* N53-1 grown in TSB with 1% glucose (I) with 5% NaCl (II), resuspended in 0.9% NaCl with 10 mM glucose during treatment with water (♦), 0.0015% (■), 0.0031% (▲) and 0.0062% (—) Incimaxx DES. The viable counts are average of duplicate determinations. Error bars are based on standard deviations from the duplicate determinations. pH_i are averages based on at least 40 single cells and error bars indicate population variation. Arrows indicate that cfu were under the detection limit in the plating assay (10 cfu/ml). The results are representative of two independent experiments (Kastbjerg et al., 2009b).

Interestingly, the protective effect of pregrowth with NaCl to Incimaxx DES observed on viability (Kastbjerg and Gram, 2009) was also reflected on pH_i . pH_i of *L. monocytogenes* grown with NaCl remained at 6-6.5 when treated with Incimaxx DES at 0.0015% (Fig. 5.4 IIa). In comparison, the pH_i of 5.5 for bacteria grown without NaCl was significantly lower (Fig 5.4 Ia). The response of the bacterial population when grown with 5% NaCl was homogenous and no sub-populations were detected.

Due to limitations of the probe at low external pH, the pH_i measurement could not be related directly to viability. Hence, we used viable counts. These remained constant over time when *L. monocytogenes* was treated with water, whereas disinfection with 0.0015% Incimaxx DES caused only a minor decrease in viability (Fig 5.4A). Despite the low pH_i of 5 to 5.5 caused by 0.0015% Incimaxx DES, the stressed bacterial cells were able to recover and grow on agar as in accordance with Shabala et al. (2002). Hence, pH_i was a more sensitive measure of adverse effects on *L. monocytogenes* than viability, since a concentration of Incimaxx DES (0.0015%) that did not significantly affect cell counts had

a marked effect on pH_i. Similarly, the ability to maintain a pH gradient was largely affected by BAC and hydrogen peroxide before a major loss in viability (according to plate counts) was detected (Luppens et al., 2003). This indicates that antibacterial components may clearly affect bacterial cells even at levels where no effect is seen on viable counts making further studies of the impact of sub-inhibitory stress on *L. monocytogenes* in relation to e.g. gene-expression and virulence relevant.

Although FRIM could detect different sub-populations in response to bacteriocins (Budde and Jakobsen, 2000; Hornbæk et al., 2006), one may speculate that a minor fraction of cells could be more tolerant to disinfectants which may not be detected with FRIM. However, the viable counts obtained during exposure to the highest concentration of disinfectant do not indicate the presence of a more tolerant population (Fig. 5.4A). Similarly, complete killing of three populations of *P. aeruginosa* cells, including stationary and logarithmic planktonic cells, and biofilm cells, by peracetic acid (400 µg/ml) were observed with viable counts, whereas presence of a surviving fraction were detected in all three populations during exposure to increasing concentrations of different antibiotics (Spoering and Lewis, 2001).

Despite the need of high cell numbers it could be interesting to determine if FRIM might detect differences on single cell level in a population during exposure to antibiotics. This might be possible by the use of the *E. coli* strain that produces a higher amount of persisters (Balaban et al., 2004). Furthermore, FRIM could be a useful method for studying co-existence of persistent and presumed non-persistent strains.

5.4 Further considerations on persistence studies

L. monocytogenes is generally sensitive to disinfectants and can be eliminated with in-use concentrations. However, survival of the disinfectant process is common both for persistent and sporadic isolates. As the persistent strains can be isolated repeatedly over a longer time period and they are not brought into the production environment from outside, it seems most likely that they have a specific trait that make them able to survive better.

So far no specific trait has been recognized that could explain the persistence of certain sub-types of *L. monocytogenes*. Different model systems have been used to clarify this issue, most of them only studying one trait at a time. One may speculate, if the ability to persist is a conjunction of more factors at once. Therefore, more complex studies where strains are exposed to several stresses at a time or several stresses in succession may be highly relevant. However, the difficulties in finding the right value or concentration when applying such stresses makes such studies challenging.

Another way to study the persistent trait could be through the use of genetic comparisons to thereby identify differences between persistent and presumed non-persistent *L. monocytogenes* strains that could lead to the identification of phenotypic differences. When plasmids were extracted from 20 *L. monocytogenes* strains representing both persistent and sporadic isolates, the strains fell into four groups with respect to the incidence and size of plasmids isolated. However, no association was seen between persistence and the presence or size of plasmids, since the persistent molecular sub-types fell into two different groups also including strains isolated on sporadic basis (Earnshaw and Lawrence, 1998). The sequence of the *inlA* gene of the persistent RAPD 9 strains used in the present study has been compared to the *inlA* gene of other presumed non-persistent strains. This has shown that RAPD type 9 strains, specifically, have mutations in *inlA*, which might explain the reduced ability of these strains to invade Caco-2 cells and kill *Caenorhabditis elegans* and *Drosophila melanogaster* (Jensen et al., 2008; A. Jensen, 2009 DTU Aqua, personal communication). InlA is one of many surface proteins, and one might speculate if other mutations could be found in the surface proteins of the persistent *L. monocytogenes* sub-types leading to the ability of these bacteria to persist in the food processing environment.

5.5 Conclusions on chapter 5

Classical techniques measuring physiological response determine average values across an entire population. Hence, single-cells differences are masked. FRIM is a useful microscope method for studying the effect of different stresses on the same single cells over time by measuring the effect on the physiological parameter, pH_i. A disinfectant based on peracetic acids caused a homogenous and pronounced decrease in pH_i. Hence, so far it seems likely that a disinfectant in sub-lethal concentrations affects the population of a persistent sub-type of *L. monocytogenes* equally and that sub-populations of higher tolerance to disinfectants are not present.

6 Virulence regulation in *Listeria monocytogenes*

L. monocytogenes has both a saprophytic and a parasitic lifestyle. As a saprophyte it is well adapted for survival in soil and decaying vegetation, whereas it as parasite infects animals and humans and causes the serious illness listeriosis. Under saprophytic conditions outside of the host, most of the proteins encoded by virulence genes are not needed. On the other hand, *L. monocytogenes* must be able to sense when it is within a host and thereby up-regulate the expression of virulence factors. The switch from the environmental bacterium to a potentially deadly pathogen appears to be mediated through complex regulatory pathways that modulate the expression of virulence factors in response to physico-chemical factors. The present chapter addresses the symptoms and incidence of listeriosis, and describes the intracellular life style of *L. monocytogenes*, including the virulence factors involved. Furthermore, it describes how the virulence factors are regulated by PrfA and physico-chemical factors.

6.1 Listeriosis

L. monocytogenes causes listeriosis which can be a non-invasive disease, but primarily occurs in an invasive form. The non-invasive form is a self-limiting acute gastroenteritis in immunocompetent persons, whereas the invasive form often affects those with a severe underlying disease or condition, e.g. immunosuppression and HIV/AIDS, pregnant women, unborn or newly delivered infants, and the elderly. The clinical signs of the invasive form are flu-like illness, septicaemia, infection of the central nervous system including meningitis, and abortion in pregnant women (Kreft and Vázquez-Boland, 2001; Swaminathan and Gerner-Smidt, 2007)

Ingestion of *L. monocytogenes* is likely to be a very common event, given the ubiquitous distribution of these bacteria. However, the incidence of human listeriosis is low. Only 0.3 cases per year per 100,000 of the population of the whole EU were observed in 2007 (EFSA, 2009). Denmark reported the highest incidence of 1.1 cases pr 100,000 persons due to 58 cases, followed by Finland, Sweden and Luxemburg with incidences of 0.6 to 0.8. A general increase in human cases of listeriosis has been seen in Europe and Denmark from 2003 to 2007, and specifically in Denmark the number of cases has doubled from 29 in 2003 to 58 in 2007, mainly affecting the elderly. The reason for this increase is

unknown. One may speculate that it could be due to a overall increase in the number of elderly. Also, the general changes in eating habits to consumption of more RTE products could contribute to the increased incidence.

Although infrequent as compared to other foodborne pathogenic bacteria, listeriosis is a severe infection and has an average case-fatality rate around 30% (Table 6.1) (Adak et al., 2002; Mead et al., 1999; EFSA, 2009). In the Canadian outbreak in summer 2008, the case-fatality rate was as high as 39% (Anonymous, 2009a). This is a markedly higher fatality rate than seen for other foodborne pathogens (Table 6.1), which makes the control of *L. monocytogenes* very important.

Table 6.1. Morbidity and mortality due to indigenous food borne disease caused by pathogens under surveillance in England and Wales in 2000 (modified after Adak et al., 2002).

Pathogen	Cases		Death		Death/cases (%)
	No	%	No	%	
<i>Campylobacter</i>	359,466	56.6	86	21.1	0.02
<i>Cl. perfringens</i>	84,081	13.3	89	21.9	0.1
<i>Yersinia spp</i>	45,144	7.1	1	0.2	“0”
<i>Salmonella</i>	41,616	6.6	119	29.2	0.29
<i>Bacillus</i>	11,144	1.8	0	0	0
<i>S. aureus</i>	2,276	0.4	0	0	0
<i>E. coli O157</i>	995	0.2	22	5.4	2.2
<i>L. monocytogenes</i>	194	< 0.1	68	16.7	35
Others	89,652	14	22	5.4	0.02
All	634,568	100	407	100	-

6.2 Infection cycle

L. monocytogenes is able to cross the intestinal barrier, the blood-brain barrier, and the placental barrier, and this is essential for its human pathophysiology. Briefly, *L. monocytogenes* enters the human host as a food contaminant and adheres to and crosses the intestinal epithelium. The organisms reach, via the lymph and blood, the lymph nodes, spleen, and liver. Most of the bacterial load accumulates in the liver. Normally, an immune cascade involving neutrophils, macrophages and T-cells will eliminate *L. monocytogenes* from the liver. However, if the infection is not controlled by an adequate immune response, *L. monocytogenes* proliferates unlimitedly, which may result in the release of the bacterium into the circulation. Due to the ability of the bacterium to cross the blood-brain and the placental barrier, it can cause meningitis, and in the pregnant woman stillbirth and abortion (reviewed by Vázquez-Boland et al., 2001).

During its infection of the human host, *L. monocytogenes* uses its facultative intracellular parasitic life style to invade and replicate in macrophages but also in various types of cells that are not normally phagocytic including epithelial cells, fibroblast, hepatocytes, endothelial cells, and various types of nerve cells, including neurons (Portnoy et al., 1988; Vázquez-Boland et al., 2001). In the interior of these cells, *L. monocytogenes* has a common and well-described life cycle (Fig. 6.1).

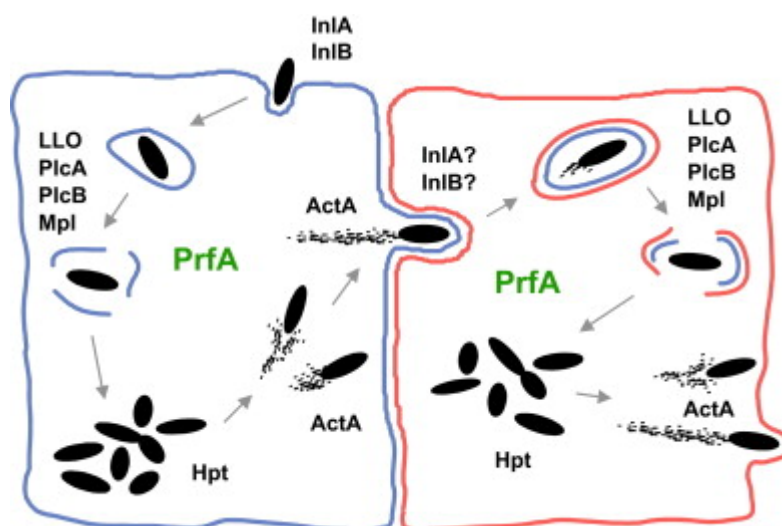


Fig. 6.1. Schematic representation of the intracellular life cycle of *L. monocytogenes*. The different proteins involved are indicated near each of the phases of the cycle in which they are involved (copied from Scortti et al., 2007).

L. monocytogenes adheres to the surface of the eukaryote cell and penetrates into a host cell in an internalization vacuole. The internalization vacuole is lysed by bacterial enzymes, and *L. monocytogenes* escapes into the host cell cytoplasm, where the bacteria proliferate and produces an actin tail. This is used by *L. monocytogenes* to propel itself in the cytosol and to invade neighbouring cells. After invasion, the bacterium is surrounded by a double-membrane, which is then lysed, allowing a new intracellular infection cycle in adjacent cells (Tilney and Portnoy, 1989). This way of cell-to-cell spread facilitates propagation of the bacterial infection without exposure to the humoral immune response of the host.

6.3 Virulence genes

L. monocytogenes uses a range of virulence factors during its infectious, intracellular life style. Most of them are encoded on a 9-kb pathogenicity island known as LIPI-1 (Listeria pathogenicity island 1) (Portnoy et al., 1992; Kreft and Vázquez-Boland, 2001) (Fig 6.2). This chromosomal locus comprises the following genes *prs*, *prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*, *ldh*. The *prs* and *ldh* genes are housekeeping genes encoding phosphoribosyl

pyrophosphate synthetase and lactate dehydrogenase, respectively (Gouin et al., 1994; Vazquez-Boland et al., 1992), and the other genes on LIPI-1 are encoding different proteins involved in the intracellular life cycle of *L. monocytogenes*. These are all regulated by PrfA, which also regulates its own transcription (Chakraborty et al., 1992; Leimeister-Wächter et al., 1990; Mengaud et al., 1991b). PrfA also regulates the expression of a number of other genes dispersed on the chromosome encoding proteins involved in the intracellular life cycle. These include the internalin locus with the *inlAB* genes (Gaillard et al., 1991) and the *hpt* gene (Chico-Calero et al., 2002). The genes and their products will be described in the following sections.

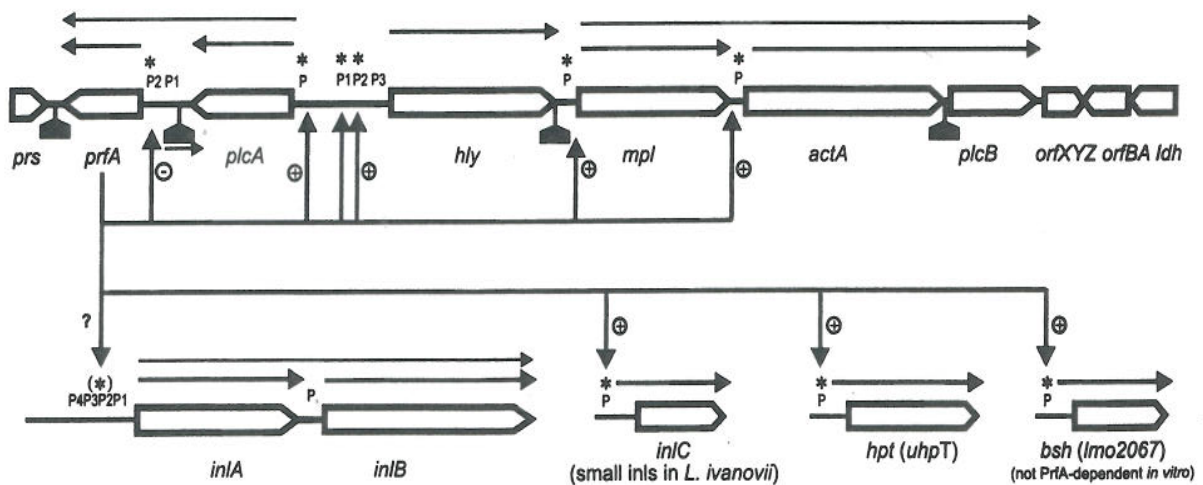


Fig. 6.2. Structure and transcriptional organization of the PrfA-dependent virulence gene cluster LIPI-1 and of other PrfA-dependent virulence genes in *L. monocytogenes* (and *L. ivanovii*). Genes and products: *prs*, phosphoribosyl synthase; *prfA*, positive regulatory factor A; *plcA*, phosphatidylinositol-specific phospholipase C; *hly*, listeriolysin O; *mpl*, metalloprotease; *actA*, actin-polymerization protein; *plcB*, broad-range phospholipase C (lecithinase); *orfXYZ* and *orfBA*, open reading frames of unknown function; *ldh*, lactate dehydrogenase; *inlA* and *inlB*, large cell-wall-bound internalins A and B; *inlC*, small secreted internalinC; *hpt*, hexose phosphate transporter; *bsh*, bile acid hydrolase; P, promoter. An asterisk above indicates the presence of a PrfA box within the promoter. Thin arrows above the gene symbols indicate the different transcripts. Arrows below with a minus or plus sign indicate transcriptional induction or repression, respectively, by PrfA. Filled pentagons show the additional open reading frames present in LIPI-1 of *L. seeliger* (copied from Goebel et al., 2006).

6.3.1 The *hly* gene

The hemolysin gene, *hly*, was the first virulence determinant to be identified and sequenced in *Listeria* spp. (Mengaud et al., 1987; Mengaud et al., 1988). Its role in virulence was recognized by transposon mutagenesis resulting in isogenic hemolysin mutants of *L. monocytogenes*, which were less virulent in mice as compared to wildtype strains (Gaillard et al., 1986). Complementation by introducing the cloned gene on a plasmid into a strain containing a structural gene mutation restored wild type virulence (Cossart et al., 1989). *Hly* is predominately transcribed from two PrfA-dependent promoters, P1 and P2 (Mengaud et al., 1989). However, also a third promoter that is PrfA-

independent has been detected (Domann et al., 1993). The *hly* transcript is a monocistronic unit of 1.8 kb (Mengaud et al., 1989; Bohne et al., 1994).

The *hly* gene is encoding the haemolysin listeriolysin O, LLO, which is essential for the bacterial escape from phagosomes (Bielecki et al., 1990). LLO is a member of the pore-forming cholesterol-dependent cytolysins (CDCs) that are produced by several pathogenic species within the gram-positive genera like *Clostridium*, *Streptococcus*, *Listeria* and *Bacillus*. As the CDCs have an absolute requirement for cholesterol in the target membrane for pore formation, the lack of sterols in the bacterial membranes, unlike eukaryotic membranes, protect them from the cytolytic activity of the CDCs (reviewed by Schnupf and Portnoy, 2007).

6.3.2 The *plcA* and *plcB* gene

Adjacent to *hly* and transcribed divergently is the *plcA* gene (Leimeister-Wächter et al., 1991; Mengaud et al., 1991a) (Fig. 6.2). *L. monocytogenes plcA* is both transcribed monocistronically as RNA transcript of approximately 1.2 kb and together with *prfA* in a bicistronic RNA transcript of approximately 2.2 kb (Mengaud et al., 1991b). *plcA* encodes a phosphatidylinositol-specific phospholipase C (PI-PLC or PlcA) that is able to cleave glycosyl-phosphatidylinositol-anchored proteins (Mengaud et al., 1991a). The phospholipase is involved in lysis of the phagosomes (Camilli et al., 1993). It only has a minor individual role in lysis but acts synergistically with the other phospholipase, PlcB and LLO (Marquis et al., 1995).

Downstream of *hly* is the lecithinase operon that comprises the genes *mpl*, *actA* and *plcB* and three open reading frames of unknown function (Mengaud et al., 1991c) (fig 6.2). The *plcB* gene is both transcribed on a *mpl-actA-plcB* 5.7 kb RNA transcript and also on a bicistronic *actA-plcB* RNA transcript (Mengaud et al., 1991c, Vázquez-Boland et al., 1992). The *plcB* gene encodes the phospholipase PC-PLC or PlcB that has a broad substrate spectrum since it is capable of hydrolyzing all of the major mammalian cell phospholipids (Goldfine et al., 1993). It is secreted as an inactive proenzyme that is activated in broth culture by proteolytic cleavage of the Mpl metalloprotease encoded by the *mpl* gene (Poyart et al., 1993; Raveneau et al., 1992). However, *in vivo* studies have shown that intracellular, activation of proPlcB is mediated by two pathways; one with MPL and one with a cysteine protease (Marquis et al., 1997). PlcB is both required in escape from the primary vacuole and are also contributing to lysis of the two membranes that enclose the bacteria after direct cell-to-cell spread (Marquis et al., 1995; Vázquez-Boland et al., 1992).

6.3.3 The *actA* gene

When observing the stages in the entry, growth, movement and spread of *L. monocytogenes* in macrophages by electron microscope, Tilney and Portnoy (1989) found that once released within the cytosol, *L. monocytogenes* became encapsulated by short actin filaments. The actin filaments were rearranged during proliferation of the bacteria and it formed a long tail (5 μ m), extending from only one end of the bacterium. This tail is essential in cell-to-cell spread.

By analyzing a transposon mutant defective in cell-cell spreading and with reduced virulence in mice, the *actA* gene was identified (Domann et al., 1992; Kocks et al., 1992). The gene is placed between the *mpl* and the *plcB* gene in LIPI-1 (Fig 6.2.) and co-transcribed either from the *mpl* promoter with *mpl* and *plcB* or from a second promoter presented upstream from *actA* (Mengaud et al., 1991c; Vazquez-Boland et al., 1992). It is encoding the ActA protein, a surface protein that is essential for the bacteria-mediated polymerization of actin filaments (Kocks et al., 1992). ActA is the primary protein responsible for this activity, since ActA expression in *L. innocua* or its coating on latex beads enables actin polymerization (Camron et al., 1999; Kocks et al., 1995). Besides its primary role in intracellular movement, ActA is involved in adhesion and invasion of *L. monocytogenes* into eukaryotic cells (Alvarez-Dominguez et al., 1997; Suarez et al., 2001).

6.3.4 The *inlAB* genes

Besides the genes in the LIPI-1 cluster, a second cluster is also required for invasion and intracellular replication of *L. monocytogenes*. This cluster contains an operon with two genes, *inlA* and *inlB* (Gaillard et al., 1991), and is located on the chromosome (Glaser et al., 2001) (Fig 6.2). The operon is controlled by a complex regulatory region located upstream of *inlA* containing four promoters of which one is PrfA-dependent (Dramsı et al., 1993; Lingnau et al., 1995). Also, a promoter is found in the intergenic region between the *inlA* and *inlB* genes (Lingnau et al., 1995). Northern blots detected three transcripts. One 5 kb transcript comprising the whole *inlAB* operon, a 2.9 kb *inlA* transcript and a 1.9 kb *inlB* transcript (Dramsı et al., 1993; Lingnau et al., 1995).

inlA and *inlB* encode for two surface proteins internalin A and B, respectively, that are required for *L. monocytogenes* invasion of epithelial cells (Lingnau et al., 1995). Both proteins have an amino-terminal leucine-rich repeat domain followed by a conserved inter-repeat domain, several other repeats and a carboxy-terminal region. In InlA, this carboxy-terminal region is a LPXTG motif, which mediates covalent binding to the cell wall

peptidoglycan. In InlB, the carboxy-terminal region is a glycine/tryptophan-rich (GW) module, which mediates electrostatic interactions to the lipoteichoic acids on the bacterial cell wall (Cabanes et al., 2002; Gaillard et al., 1991; Jonquieres et al., 1999). However, both proteins are not only associated with the cell surface but also found in culture supernatants of *L. monocytogenes* (Dramsi et al., 1993; Braun et al., 1997).

The two proteins have different cellular receptor molecules giving *L. monocytogenes* the ability to invade many different cell-types. The InlA-receptor molecule is E-cadherin, a cell-cell adhesion molecule expressed in epithelial tissues, primarily in the skin, the liver, and the digestive track (Mengaud et al., 1996). InlB-dependent internalization is mediated by the Met receptor tyrosine kinase, expressed by endothelial cell in the brain, placenta villi, and umbilical cord, and the receptor for gC1qR (the globular head of the complement C1q molecule), expressed on a wide range of cell types including lymphocytes, neutrophils, hepatocytes, and endothelial cells (Shen et al., 2000; Braun et al., 2000).

6.3.5 The *hpt* gene

The *hpt* gene was identified when screening the genome of *L. monocytogenes* for the binding site of PrfA in target promoters (Chico-Calero et al., 2002). A Δhpt mutation had no effect on the growth of *L. monocytogenes* in broth culture; however, the mutant growth was clearly impaired in various mammalian cell types and had attenuated virulence in mice. The gene encodes a hexose phosphatase translocase that is specifically involved in the intracellular proliferation of *L. monocytogenes*.

6.3.6 Other virulence genes

The virulence genes and their products mentioned so far are all involved in the intracellular life style of *L. monocytogenes*. They have been identified by the use of genetic tools like transposon mutagenesis. Similarly, this technique has been used to screen for mutants impaired in virulence in order to find other genes encoding proteins suggested to be involved in virulence. Thereby, the *fbpA* gene was identified, encoding a surface protein that binds fibronectin and mediates adherence, and also modulates the protein level of LLO and PlcB (Dramsi et al., 2004).

Comparative genomics is another way of identifying new virulence determinants. The comparison of the genomes of *L. monocytogenes* and the non-pathogenic species *L. innocua* led to the identification of 270 DNA fragments absent in *L. innocua* (Glaser et al., 2001). One of these fragments is the *bsh* gene encoding a bile salt hydrolase that contributes to survival of *L. monocytogenes* in the intestinal lumen (Dussurget et al., 2002).

Others are Auto, an autolysin, and Vip, an LPXTG-anchored protein, both involved in entry into host cells (Cabanès et al., 2004; Cabanès et al., 2005). An unexpected high number of genes encoding surface proteins were detected in the genome comparison. These include internalin-like proteins of which some have a role in virulence e.g. InlC. (reviewed by Bierne et al., 2007).

6.4 PrfA, master regulator of virulence

PrfA (positive regulatory factor A) is the prime virulence regulator in *L. monocytogenes* and is directly involved in control of virulence gene expression. Other regulators also influence virulence gene expression e.g. SigB, an important stress-regulator in *L. monocytogenes* that regulates PrfA and *inlAB* expression (Chaturongakul et al., 2008). However, here I will focus on PrfA.

The *prfA* gene was identified by genetic analysis of a hemolysin-negative mutant and was found to be located downstream from *plcA* in LIPI-I (Fig 6.2) (Leimeister-Wächter et al., 1990). The *prfA* gene is transcribed monocistronically from two promoters located in the *plcA-prfA* intergenic region, giving rise to two transcripts of 0.8 and 0.9 kb, and in addition as a bicistronic *plcA-prfA* mRNA from the *plcA* promoter (*PplcA*) giving rise to a 2.1 kb transcript (Camilli et al., 1993; Freitag et al., 1993; Mengaud et al., 1991b). The gene encodes the 27 kDa polypeptide PrfA and its absence in a Δ *prfA* mutant caused the strain to be avirulent and unable to grow in host tissues (Chakraborty et al., 1992; Leimeister-Wächter et al., 1990).

PrfA belongs to the Crp/Fnr family of transcription activators. Although the overall similarity of PrfA with the Crp (cyclic AMP receptor protein) of *E. coli* is low on the primary sequence level, there are significant sequential and structural similarities (Fig. 6.3) (Lampidis et al., 1994; Sheehan et al., 1996). The Crp protein has therefore been used to elucidate the nature of PrfA. Crp exists in two functional states: weakly active in the native form, and highly active after a conformational change caused by the cAMP-cofactor. However, hyperactive mutants of Crp, denoted Crp*, contain amino acid substitutions that cause a similar high-level activity as a cAMP-complexed Crp, but without the need for a co-factor. Like Crp, PrfA can exist in two functional states. The evidence is based on work with *prfA** mutants which express a hyperactive PrfA* that causes constitutive overexpression of the PrfA-dependent genes, and are no longer affected by environmental conditions (Ripio et al., 1997; Sheehan et al., 1996; Vega et al., 2004). This, together with the structural similarities of PrfA with the co-factor binding domain in Crp, suggests that

PrfA is also allosterically activated by a co-factor (Reviewed by Scotti et al., 2007). However, a cofactor has not been identified.

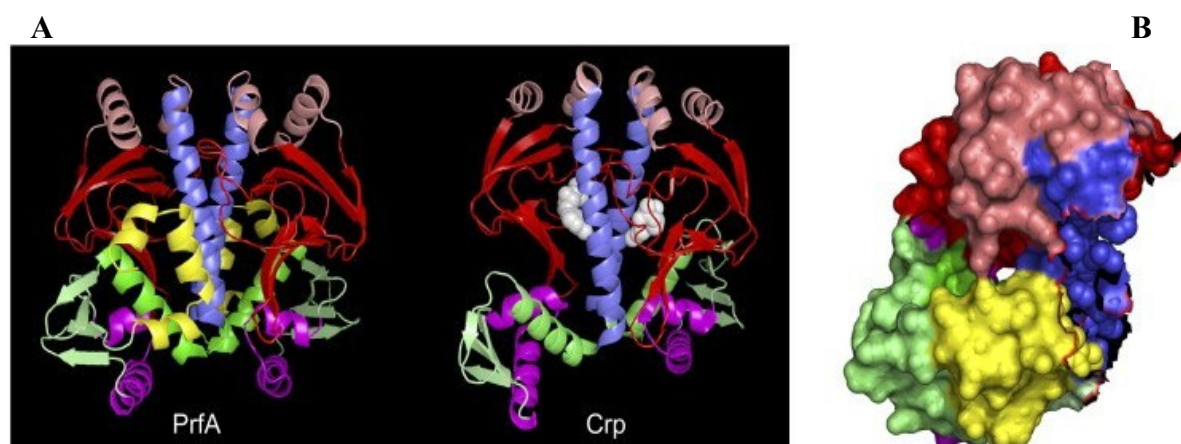


Fig. 6.3. Structural similarities between PrfA and Crp/Cap. (A) Comparison of the crystal structures of the PrfA and Crp dimers; the Crp structure shows the cAMP molecule (white spheres) inserted in each of the two β (jelly)-rolls of the dimer. **(c)** Combined surface and space-fill representation of the “left” PrfA monomer (PDB-ID 2BEO) showing the solvent-accessible tunnel at the interdomain cleft; a similarly located tunnel gives access to the cAMP-binding pocket in Crp (copied from Scotti et al., 2007).

Complementation experiments have shown that all virulence genes at LIPI-I requires PrfA for expression including it self. Other virulence genes are also regulated by PrfA including *inlAB* and *htp* as mentioned earlier, but also *bsh*, encoding a bile salt hydrolase (Dussurget et al., 2002), and *inlC* (Engelbrecht et al., 1996). More PrfA regulated genes are likely to be discovered as a screening of the genome of *L. monocytogenes* for binding sites for PrfA in target promoters revealed new putative members of the PrfA regulon (Chico-Calero et al., 2002). PrfA-dependent transcription is influenced both by regulation of PrfA itself, of promoter configuration of the virulence gene promoters, and of environmental factors, which will be described in the following sections.

6.4.1 Regulation of PrfA expression and translation

The *prfA* gene is transcribed both monocistronically from its own promoters but also bicistronically from the PplcA. During exponentially growth, the bicistron becomes predominant, whereas the monocistronic transcripts of both *prfA* and *plcA* dominate in late exponential growth. In stationary phase, the overall amount of *prfA* transcripts drops (Mengaud et al., 1991b). This and other studies have led to hypothetical models of transcriptional regulation of the *prfA* gene (Camilli et al., 1993; Freitag et al., 1993; Freitag & Portnoy, 1994; Scotti et al., 2007; Vega et al., 2004; Wong and Freitag, 2004). The model described by of Scotti et al. (2007) relies on the capacity of PrfA to undergo allosteric transition upon interaction with a co-factor, as mentioned in section 6.4. Briefly, it describes that basal level of monocistronic *prfA* transcripts are made from the *prfA*

promoters and appear to be sufficient to stimulate the *PplcA*, when a co-factor is present (Fig. 6.4). Translation of the *prfA/plcA* bicistron leads to elevated amounts of PrfA protein, thus activating the transcription of other PrfA-dependent genes and further increasing bicistron synthesis. This autoregulatory loop ensures high levels of PrfA, which is essential for a productive infection (Camilli et al., 1993; Mengaud et al., 1991b). The model proposes that the autoregulatory loop is controlled by an auto-repressor that so far is unknown. Others have proposed that P2*prfA* is involved in a negative autoregulation of the *prfA* gene (Bubert et al., 1997; Freitag et al., 1993; Freitag and Portnoy, 1994), but other studies again reported no evidence of the involvement of P2*prfA* in PrfA autorepression (Sheehan et al., 1995; Sheehan et al., 1996).

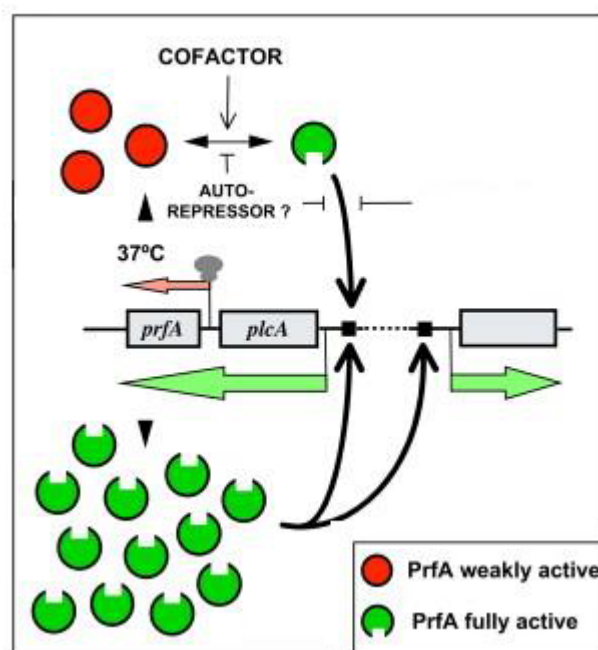


Fig. 6.4. PrfA regulation model with representation of control levels and underlying mechanisms. Central in the model is the capacity of PrfA to undergo allosteric transition from weakly active to highly active conformation upon interaction with a putative low-molecular weight cofactor. PrfA system in its active state: a cofactor binds to PrfA and increases the affinity of the regulatory protein for its target sequence (the PrfA box, represented by black squares); the highly active PrfA causes the synthesis of more PrfA – which immediately adopts the highly active conformation because the cofactor is present – via the positive autoregulatory loop generated by the PrfA-dependent bicistronic *plcA-prfA* transcript, resulting in high levels of induction of the PrfA virulence regulon. Full induction of the PrfA regulon is achieved if the environmental temperature is 37 °C, the cofactor is present and repressor signals are not acting on the system (modified from Scortti et al., 2007).

Besides of transcriptional control, *prfA* is controlled translationally by temperature (Johansson et al., 2002). At low temperatures (< 30 °C) bicistronic *prfA* expression is lower than at 37 °C, whereas monocistronic *prfA* is equally well transcribed (Leimeister-Wächter et al., 1992). This coincides with the absence of the PrfA protein (Johansson et al., 2002; Leimeister-Wächter et al., 1992; Renzoni et al., 1997), which is not due to a general

untranslability of the *prfA* monocistronic messenger (Renzoni et al., 1997). Instead, Johansson et al. (2002) found that the 5'-untranslated region (5'-UTR) mRNA preceding *prfA* from P1*prfA* forms a secondary structure that mask the Shine-Dalgarno (SD) site, the ribosome binding region, thereby preventing ribosome binding and consequently, translation. At higher temperatures (37 °C), this secondary structure is partially disrupted, which enables binding of the ribosome and translation. Hence, temperature might function as a efficient signal for activation of virulence genes when the bacteria are in the host. In addition to the existence of mechanisms that control *prfA* expression and translation, the PrfA activity, and hence virulence gene expression, is affected on posttranscriptional level by physico-chemical factors, and these are further described in section 6.5.

6.4.2 Regulation by PrfA

PrfA regulates the different virulence genes by direct activation of transcription through a target sequence, the “PrfA-box”. This is a 14-bp region of dyad symmetry centered at position -41 relative to the transcriptional starting site and partially overlaps the -35 promoter regions (Dramsi et al., 1993; Freitag et al., 1992; Kreft and Vázquez-Boland, 2001; Mengaud et al., 1989; Vázquez-Boland et al., 1992). However, there is a hierarchy in the *prfA*-regulated promoters as activation by PrfA is more efficient at promoters which possess a perfectly symmetrical PrfA box like *PplcA* or *Phly*, than at promoters which have substitutions such as *PactA* (1 sub.), *Pmpl* (1 sub.) and *PinLAB* (2 sub.) in the PrfA box (Sheehan et al., 1995). This differential response is due to differences in the affinity of PrfA for the structurally different palindromes (Vega et al., 2004). Another feature that also appears to determine the response pattern of PrfA-regulated promoters is their affinity for RNA polymerase (Vega et al., 2004).

Other factors also contribute to differential expression of the virulence genes. The amount of GTP and ATP present seems to determine if *in vitro* transcription initiates from the PrfA-dependent or PrfA-independent promoters, when more promoters are present in front of the gene (Luo et al., 2004). Also, the confirmation of the region around the -10 RNAP element influences the strength of the PrfA binding (Luo et al., 2005). Furthermore, as for *prfA*, the translation of the virulence genes *inlA*, *hly* and *actA* is controlled by their 5'-untranslated region that can form a secondary structure. However, contrary to the *prfA*, the signal for enhanced translation of these other virulence genes is not known (Loh et al., 2006; Stritzker et al., 2005).

6.5 Regulation of virulence genes by physico-chemical factors

Several environmental factors affect the expression of virulence genes in *L. monocytogenes*. These are factors relevant in different habitats of *L. monocytogenes*, including food commodities and may very well function as signals for the bacteria in order for it to sense if up- or down-regulation of virulence factors is needed dependent of its habitat. Two of the factors, carbohydrates and charcoal, have been studied more intensively in order to determine how they affect virulence regulation.

Repression of PrfA dependent gene expression is observed when *L. monocytogenes* is grown on fermentable carbohydrates transported by the phosphoenolpyruvate-sugar phosphotransferase system (PTS), such as glucose, fructose, mannose or cellobiose (Milenbachs et al., 1997; Stoll et al., 2008). Down regulation only occurs if the amount of carbohydrate is sufficient to promote bacterial growth, suggesting that the underlying mechanism is related to carbon catabolite repression (CCR) (Milenbachs et al., 1997). However, Behari and Yongman (1998b) found that CcpA, a catabolite control protein, is not involved in carbon source regulation of virulence genes in *L. monocytogenes*. Recent evidence suggest involvement of Hpr-Ser-P, a corepressor of the CcpA transcription, possibly through an indirect mechanism (Herro et al., 2005), or interaction of PrfA with a common component involved in PTS-mediated sugar uptake as e.g. the unphosphorylated PTS permease (Marr et al., 2006; Stoll et al., 2008), but still no exact mechanism has been ruled out.

On the contrary, expression of virulence genes is significantly enhanced at 37 °C, when *L. monocytogenes* is grown in culture medium supplemented with activated charcoal, leading to an increase in the amount of LLO and PlcB protein and in hemolysin and lecithinase titers (Ripio et al., 1996). The “charcoal effect” results from the adsorption of a diffusible autorepressor substance, presumably a small hydrophobic molecule, that is released by *L. monocytogenes* during exponential growth and which affects, directly or indirectly, the activity of PrfA. As the inhibitory effect of this autorepressor is bypassed in the PrfA* mutation that locks PrfA in its fully active conformation, the autorepressor might interfere with the allosteric activation mechanism of PrfA (Ermolaeva et al., 2004). However, the induction of virulence factors *in vitro* in the PrfA* strains did not increase virulence in mice (Ripio et al., 1996; Shetron-Rama et al., 2003).

Other physico-chemical parameters affect expression of PrfA and PrfA-dependent genes, but the mechanisms have not been studied further. Low iron and other nutrient stress conditions limiting growth induce virulence gene expression of *prfA*, *plcA*, *hly* and *actA*

(Böckmann et al., 1996; Conte et al., 1996; Sokolovic et al., 1993), whereas *inlAB* is up-regulated in iron rich media correlating with an enhanced invasive capability (Conte et al., 1996). The differences in iron modulation may reflect differences in the need of the bacteria to express these virulence factors, since *InlAB* is used extracellular while the other virulence genes are used intracellular.

Decreased availability of oxygen induces haemolytic activity and *in vivo* invasiveness (Andersen et al., 2008; Kouassi and Shelef, 1995). The invasiveness was increased both *in vitro* in the human intestinal epithelial Caco-2 cells and *in vivo* in guinea pigs for *L. monocytogenes* grown under oxygen restricted conditions as compared to *L. monocytogenes* grown aerobically (Andersen et al., 2008). Although not determined, this could very likely be due to increased expression of *InlA*, since anaerobic growth enhanced production of *InlA* in *aro* mutants of *L. monocytogenes* (Stritzker et al., 2005).

The influence of NaCl on virulence expression is dependent on concentration. The haemolytic titer, a measure for LLO activity, increased when *L. monocytogenes* was grown with increasing NaCl concentration. However, a maximum level was reached at 428 mM (~2.5%), since higher concentrations decrease the haemolytic titer (Dallmier and Martin, 1990; Myers et al., 1993). Growth with 428 mM NaCl did not enhance virulence of the strain (Myers et al., 1993). Also, expression of *inlA* was upregulated during growth with 0.3 M NaCl (~1.8%) (Sue et al., 2004). Invasions studies in Caco-2 cells with *L. monocytogenes* grown with NaCl have given contradictory results. Garner et al. (2006) found that the invasion ability in Caco-2 cells was increased by growth in NaCl (2.2%) at 37 °C, but reduced by pre-exposure to gastric fluid. However, the increased invasion ability was not linked to gene expression or protein level. On the other hand, pre-growth with 5% NaCl did not enhance virulence potential of different *L. monocytogenes* strains in different virulence models including Caco-2 cells (Jensen et al., 2008). However, this discrepancy could be due to the higher NaCl concentration used.

Acid stress increases invasion of *L. monocytogenes* in cells. *L. monocytogenes*, exposed to a sublethal acidic pH (pH 5.1), showed increased invasion of Caco-2 cells, and increased survival and multiplication in macrophage-like cells relative to nonexposed bacteria (Conte et al., 2000). This was related to an altered pattern of gene regulation. Whereas overall levels of *prfA*-specific messengers were not significantly affected by pH, the pattern of *prfA* transcription was clearly pH dependent. Transcription of the *plcA* gene was downregulated, whereas monocistronic transcription was increased in acid-adapted cells. Transcription of *inlA*, *hly* and *actA* was not different in acid-adapted and non-adapted *L.*

monocytogenes. This unchanged level of *hly* is not in accordance with others that found a reduced production of LLO at pH 5 as compared to pH 7 and repressing in *hly* expression from pH 6 to 6.7 compared to pH 7 (Behari and Youngmann, 1998a; Datta et al., 1993).

Growth with preservatives affects LLO secretion differently. Nitrite reduced both growth and LLO secretion, whereas the LLO secretion was selectively inhibited by sorbate measured as hemolytic activity (Kouassi and Shelef, 1995; Mckellar, 1993). On the other hand, citrate and acetate added in sub-inhibitory concentrations enhanced LLO secretion (Kouassi and Shelef, 1995). These studies were performed as growth experiments in media, and the response might change in a food matrix.

In the food processing environment, cleaning and especially the disinfection compounds are intended to kill the bacteria. However, it may happen that the disinfection process is not adequately performed or organic debris inactivates the disinfectant as mentioned earlier, and the bacteria are therefore only exposed to sub-lethal concentrations of the active components. Whereas adaptation and resistance to the disinfectants may be less likely, the sub-lethal concentrations do stress the bacteria as discussed in chapter 5, and one may speculate how such components affects the expression of virulence genes in *L. monocytogenes*. This might affect virulence, but also other factors as changed susceptibility to antibiotics as discussed in chapter 4. In this thesis, we have as the first addressed the potential effect of disinfectants in sub-lethal concentrations on the expression on virulence genes in *L. monocytogenes*.

We developed an agar-based screening assay and used it to determine how different disinfectants affected the expression of four virulence genes in *L. monocytogenes* (Table 6.2) (Kastbjerg et al., 2009a). Basically, the agar assay relied on a *lacZ* fusion to the promoters of the virulence genes and hence, promoter activity could be detected due to hydrolyse of x-gal by β -galactosidase. Eleven different industrial compounds were tested, and we found that some compounds induced whereas others reduced expression of the *prfA*, *plcA*, *inlA* and *hly* gene, determined as promoter activity (Table 6.2). In general, compounds with the same active ingredient had the same effect on gene expression, and the effect was similar for all four virulence genes. The effects seen by Compound 1 and 7 in the agar assay were further confirmed at transcriptional level by Northern blot analyses for *prfA* and *inlA* (Fig 6.5).

Table 6.2. Effect of different disinfectants on expression of different virulence genes of *Listeria monocytogenes* (Kastbjerg et al., 2009a).

Type of disinfectant	Designation	Concentration	Down(-) or up(+) regulation of gene				
			prfA-fusion	plcA-fusion	inlA-fusion	ΔprfA inlA-fusion	hly-fusion
Peroxy compounds	Compound 1	1.25% - 40%	-	-	-	-	-
	Compound 2	0.31% - 10%	-/+	-/+	-/+	-/+	-/+
Chlorine	Compound 3	3.13% - 100%	-	-	-	-	-
	Compound 4	3.13% - 100%	-	-	-/+	-/+	-
	Compound 5	3.13% - 100%	-	-	-	-	-
Triclosan, ethanol	Compound 6	0.19% - 6%	+	no	+	+	+
Quaternary ammonium compounds	Compound 7	0.0063% – 0.2%	+	+	+	+	+
	Compound 8	0.0063% – 0.2%	+	+	+	+	+
	Compound 9	0.0063% – 0.2%	+	+	+	+	+
	(neutral pH)						
	Compound 10	0.0063% – 0.2%	+	+	+	+	+
	(acid pH)						
	Compound 11	0.0063% – 0.2%	+	+	+	+	+
	(alkaline pH)						

+ represents a induction in the expression of the virulence gene

- represents reduction in the expression of the virulence gene

-/+ represents reduction followed by induction in the expression of the virulence gene

No: neither induction nor reduction in the expression of the virulence gene

These results indicate that sublethal concentrations affect virulence gene expression in *L. monocytogenes*, and the effect depends on the active ingredients in the compounds. However, further studies are needed to elucidate, if such changes in virulence gene expression have a phenotypic outcome (e.g. changed hemolysin activity) and if it affects virulence. This could be relevant in risk analyses of the use of disinfectants especially in settings where the route of pathogen from disinfectants exposure to the host is shorter as it is in food processing settings. Also, one may speculate, as mentioned in chapter 4, if this increase in virulence gene expression detected with some disinfectants could be general on more genes, thereby leading to a selection of antibiotic resistance due to increased activity of efflux pumps, or an increase to other stress factors as food preservations compounds.

A number of environmental factors influence the expression of PrfA-dependent gene products in *L. monocytogenes*. Each of the stresses can relate to one or more of the habitats of the bacterium, and hence indicate how these conditions affect virulence gene expression. However, in order to relate this to virulence, such effects should be confirmed *in vivo*. This is important since changes in virulence expression might not always have a phenotypical consequence, and although phenotypic alterations are observed, this might

not necessarily increase virulence *in vivo* as seen with a PrfA* strains. Often, model conditions in laboratory set-ups are less complex as live conditions and hence, the alteration in gene expression might both be enhanced and counteracted by other parameters in a more complex *in vivo* system.

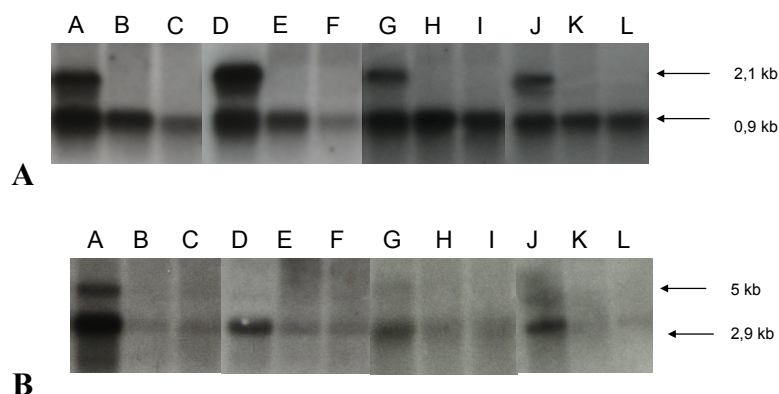


Fig. 6.5. *Listeria monocytogenes prfA* (A) and *inlA* (B) transcription measured by Northern using RNA isolated from *L.monocytognes* EGD grown in BHI-AC (0.2% charcoal) for 15 min (lanes A, B, C), 30 min (lanes D, E, F), 60 min (lanes G, H, I) and 180 min (J, K, L) with water (control) (lanes A, D, G, J), 0.125% (lanes B, E, H, K) or 0.250 % Compound 1 (lane C, F, I, L). Arrows indicates the sizes of the *plcA-prfA* (2.1 kb), *prfA* (0.8 and 0.9 kb), *inlAB* (5 kb) and *inlA* (2.9 kb) transcripts (Kastbjerg et al., 2009a).

6.6 Conclusions from chapter 6

L. monocytogenes causes listeriosis, which has a low incidence but a very high fatality rate. Through infection, *L. monocytogenes* spreads intracellularly by the use of some very well-described virulence factors. These are all regulated by the PrfA. Regulation by PrfA depends of the activity and concentration of the proteins itself, but also on the configuration of the virulence gene promoter region to which PrfA binds. However, physico-chemical factors also have an influence on virulence gene expression. In this thesis we found that industrial disinfectants affect virulence gene expression and further studies will help to determine if this has significance for *in vivo* virulence and for other biological properties.

7 Concluding remarks

Listeria monocytogenes must be considered as one of the most important food-borne pathogens. This is due to several factors: the ubiquitous nature of *L. monocytogenes*, the ability of this bacterium to survive and grow in many types of food products and the serious nature of the listeriosis infection. One of the big hurdles in controlling this pathogen is the remarkable ability of specific molecular sub-types to persist for years in the food processing environment.

Several hypotheses have been proposed to explain the persistence trait, but so far none of these have been confirmed. Work in this thesis demonstrated that *L. monocytogenes* survives drying very well when protected by organic material. This was found as a general trait for the different strains of *L. monocytogenes* and not unique to persistent types. Furthermore, it was hypothesized that specific molecular sub-types could persist due to enhanced adhesion strength to surfaces. Initial studies with AFM indicated no difference in adhesion strength between a persistent and a presumed non-persistent strain of *L. monocytogenes*. However, these studies have to be repeated to confirm the observations.

L. monocytogenes is generally sensitive to the chemical disinfectants used in the food industry, and they are killed at concentrations much lower than in-use concentrations. Inter-strain comparisons are therefore only possible when using lower concentrations of disinfectants. We found differences in sensitivity to disinfectants between strains when evaluating several *L. monocytogenes* isolates representing different origin both as planktonic, and spot inoculated and dried bacteria. However, the hypothesis that persistence of specific molecular sub-types is caused by increased tolerance to disinfectants could not be verified in this thesis. The disinfectant based on peracetic acid caused a pronounced and homogenous decrease in pH_i of a persistent sub-type, when studied on single cell level. Hence, it seems unlikely that specific molecular sub-types persist due to presence of a sub-population with decreased susceptibility or resistance to disinfectants. The apparent lack of disinfectants to control the bacterium in the production environment could be due to an insufficient disinfection process caused by exposure of a too low concentration, due to inactivation by organic debris, an unequally distribution in

the processing environment or a poor design of the equipment resulting in areas not being disinfected.

Different model systems have been used to address the persistence issue, and most of them have only considered one trait at a time. However, more complex studies are needed in order to elucidate the persistence trait. Such studies should include growth and survival in mixed culture, but also exposure to more than one stress, as the persistence trait can be caused by an ability to cope with several stresses at a time or several stresses in succession. However, the difficulties in finding the right value or concentration when applying such stresses makes such studies challenging. Explaining the persistent trait is an important riddle to solve as it may help to control the contamination of food products with *L. monocytogenes* during production.

In this thesis we found that pre-growth with 5% NaCl increased the tolerance of planktonic *L. monocytogenes* cells to a peracetic disinfectant but not to a QAC disinfectant. Furthermore, the presence of NaCl protected *L. monocytogenes* through drying, but not through disinfection of attached bacteria. Hence, it is not clear if differences in pre-growth could affect sensitivity of strains to disinfection procedures in the industry. It does however underline that environmental parameters of relevance for a particular food production should be included when studying the inactivation of pathogenic bacteria.

L. monocytogenes cells was found equally sensitive to disinfectants *per se* whether attached or suspended when calibrating model systems to include equal organic material and equal volumes and concentrations of disinfectants. Hence, the higher concentrations needed for eliminating attached bacterial cells as compared to planktonic cells is due to protection from organic material, decreased possibility for the disinfectant to penetrate the cell or cell changes only present when the cell is attached.

Sub-lethal concentrations of a peracetic disinfectant that did not affect viability of *L. monocytogenes* did stress the bacterial cells as measured by a decrease in pH_i. Also, sub-lethal concentrations of disinfectants affects expression of virulence genes in *L. monocytogenes* as determined with an agar-based assay and confirmed with Northern blot analysis. Further studies are needed to determine the phenotypic outcome and the possible effect on virulence. It is also relevant to study if exposure of *L. monocytogenes* to sub-lethal concentrations of disinfectants alters its resistance or tolerance to other stress factors, such as food preservations compounds or antibiotics.

So far, a low prevalence of resistance to disinfectants is found in *L. monocytogenes* and other food-related bacteria despite more than 100 years of use of these compounds. This indicates that if used appropriately, these compounds can effectively eliminate *L. monocytogenes*, also the persistent sub-types. In contrast, resistance to antibiotics is increasing and often observed very shortly after the introduction of the component into clinical use. The differences are caused by the differences in the spectrum of these compounds and their use. A major increase in resistance to chemical disinfectants seems unlikely as long as the products have a broad spectrum. However, it is important to follow the resistance development to disinfectants and to understand how disinfectants in sub-lethal concentrations affect bacteria to ensure that the problem will not accelerate.

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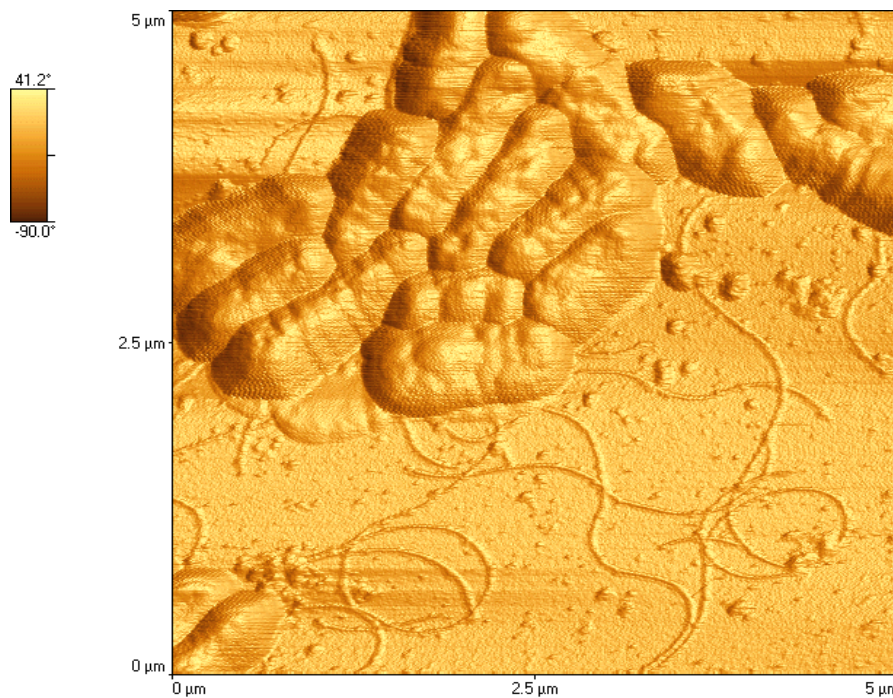
Report

Kastbjerg, V.G. and Whitehead, K.A. (2007)

Use of atomic force microscope to determine the ease of bacterial removal for a food processing persistent strain and a type strain of *Listeria monocytogenes*.

Not intended for publication.

Use of atomic force microscope to determine the ease of bacterial removal for a food processing persistent strain and a type strain of *Listeria monocytogenes*



AFM image of *L. monocytogenes* Scott A grown without NaCl spotted on silica.
Scan size: 5x5 μm .

Vicky Gaedt Kastbjerg, DIFRES, DTU (partner 21)
&
Kathryn A. Whitehead, MMU (partner 23)

June 2007

Introduction

Particular sub-types of *L. monocytogenes* are often encountered as persistent strains in food processing environments. It has been hypothesized that these bacteria may persist due to better adhesion to surfaces found in the processing environment.

Several *L. monocytogenes* auto-aggregate and adhere better to a plastic surface when NaCl (3-5%) is added to the growth medium (Jensen et al., 2007). We do not know if addition of NaCl also influences the adhesion strength of *L. monocytogenes* cells to stainless steel (SS). However, through PathogenCombat it has been demonstrated that a higher number of *L. monocytogenes* cells survive drying when spot inoculated if the organism is grown and spot inoculated with 5% NaCl.

Atomic force microscopy (AFM) can be used to measure the strength of bacterial attachment on a surface (Dufrêne, 2002). The AFM can measure forces applied in order to assess cell-cell and cell-surface interactions and the ease of removal of cells from a surface. During scanning, the perpendicular force between the tip and the surface is kept low so that the cantilever and tip move in response to changes in surface topography. However, the raster fashion of the AFM-tip can be used to displace attached cells by deliberately increasing the perpendicular tip-surface force (Boyd et al., 2002).

The ease of removal of differently sized and shaped bacteria from substrata with defined surface topographies and features has been investigated with AFM (Whitehead et al., 2006). The shape of the cell with respect to the shape of the substratum features influenced the ease of removal of the cell from the surface. It was found that *S. aureus* were removed more easily from smooth surface with increased perpendicular tip force as compared to *P. aeruginosa* cells which might be due to differences in the cell-surface contact area.

The objective of the exchange of Vicky Kastbjerg from DTU (partner 21) to Kath Whitehead MMU (partner 23) was to study adhesion strength of strains of *L. monocytogenes* using the AFM microscopy. The aim was to clarify if differences in adhesion strength of *L. monocytogenes* could be measured when comparing a persistent strain represented by N53-1 and the type strain, Scott A. The influence of growth with and without NaCl on the adhesion strength of the bacterial cells to SS was determined.

Materials and methods

Two strains of *L. monocytogenes*, a persistent strain N53-1 and a type strain Scott A, were cultivated in TSB with 1% glucose and TSB with 1% glucose and 5% NaCl at 30 °C. Cells were harvested at 3000 rpm for 10 minutes and washed three times in distilled water. The cells suspensions were adjusted to an optical density (OD) 1.0 at 540 nm, and 40 µl were spotted on a stainless steel coupon and allowed to dry for 20 hours. AFM was used to determine the ease of bacterial removal from SS by scanning the AFM tip across the retained cells under liquid using

contact mode. Scanning was performed on two distinct areas on each coupon. Attempts to remove cells of *L. monocytogenes* that were dried onto surface without further application of liquid were unsuccessful, since they could not be removed. Non-washed cells spotted on SS were also scanned. Imaging of washed cells spotted on silica was carried out in order to examine the morphology of single cells.

Results and discussion

The AFM was used to measure the strength of bacterial attachment by scanning an area under water several times in a lateral/parallel fashion. The perpendicular tip force was increased following each scan. For all samples, the remaining cell number on the substrata decreased with increasing scan number (Figure 1). This was due to the increase in perpendicular force, since scanning with an AFM tip at a constant perpendicular force over the same time range only caused a minor decrease in the cell number.

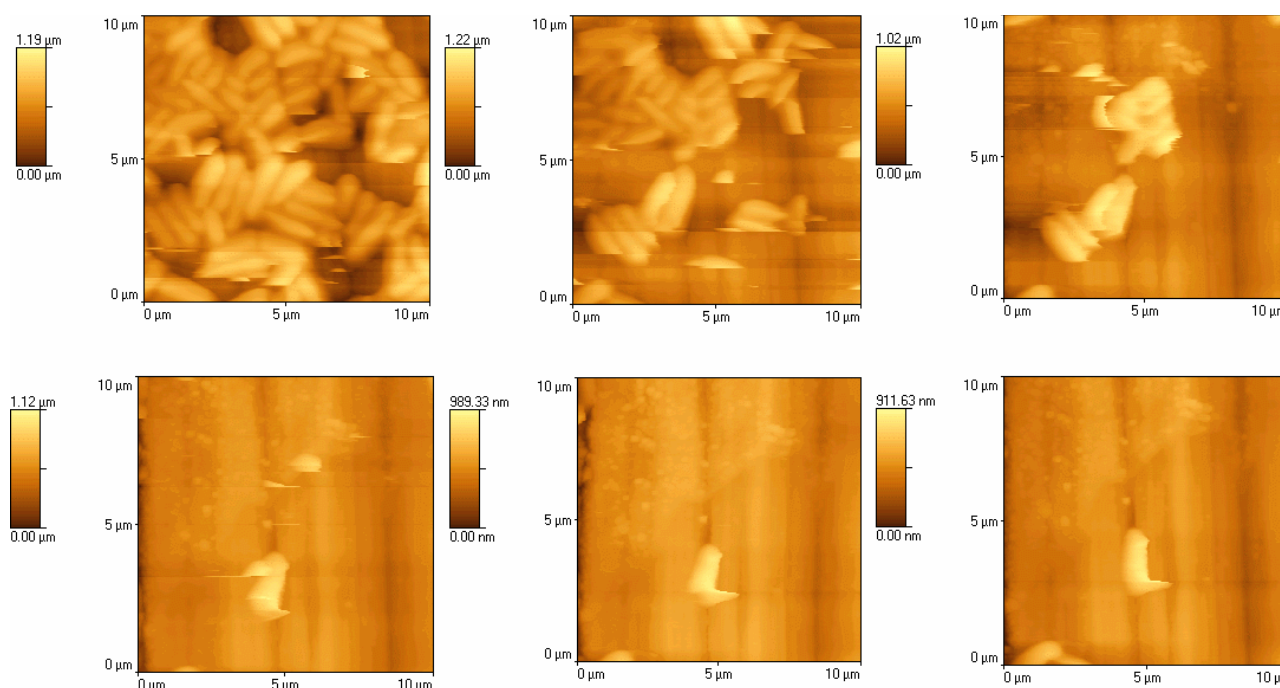


Fig 1. AFM images of Scott A grown without NaCl spotted on stainless steel with increasing perpendicular tip force. Scan size: 10 µm x 10 µm.

A difference in removal of cells was seen for Scott A grown with and without NaCl, since the number of remaining cells decreased more rapidly for cells grown without NaCl as compared to cells grown with NaCl (Figure 2). Differences in initial cell number were observed, since it was higher in the scan range for cells grown with NaCl compared to cells grown without NaCl.

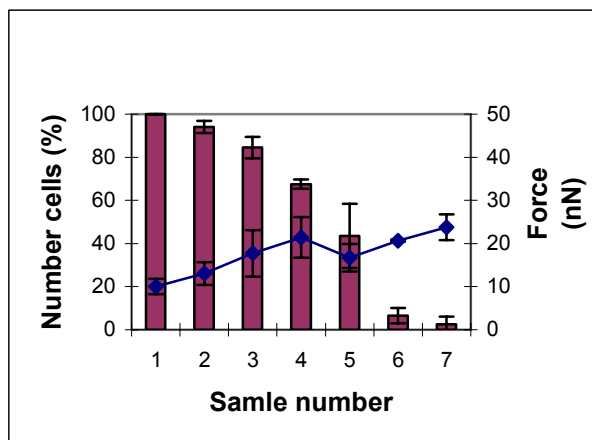
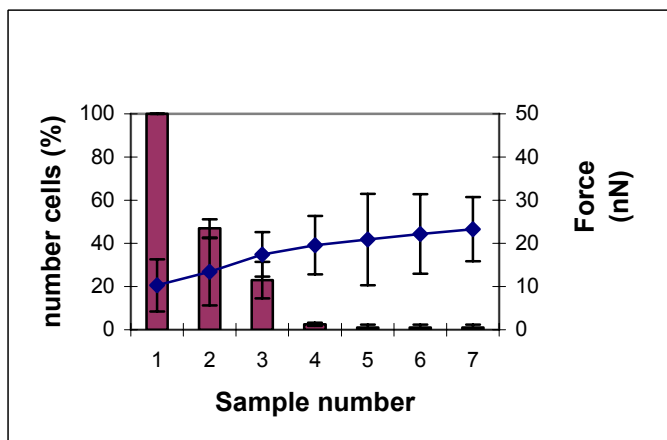
A**B**

Fig 2. Removal of Scott A grown with NaCl (A) and without NaCl (B) at increasing perpendicular force over time on SS illustrating that with increased force (line) a greater number of cells (columns) are removed from the substratum.

For N53-1 grown without NaCl it was not possible to make numerical counting of the cells due to poor image resolution of the scanning, though, it was evident that the cell number decreased with increasing force. By visual comparison of samples of N53-1 grown with and without NaCl no difference could be seen for this strain. Time constraints did not allow enough replications of scans to be able to obtain more data to measure the strength of attachment of N53-1 grown without NaCl.

Comparison of Scott A and N53-1 when grown with NaCl found that no difference in the strength of attachment were evident between the strains (Figure 2a and 3). However, with a greater number of scans it may be possible to statistically test the results.

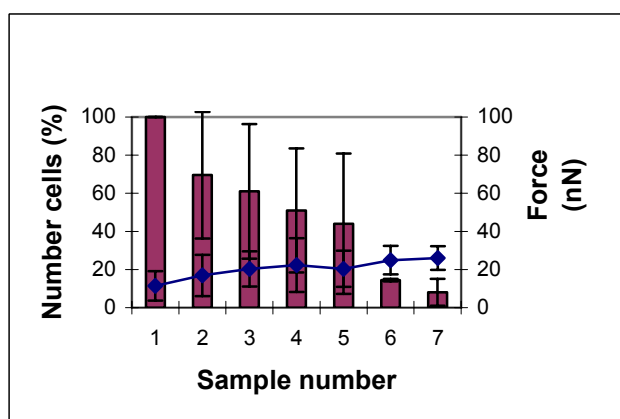


Fig 3. Removal of N53-1 grown with NaCl at increasing perpendicular force over time spotted on SS illustrating that with increased force (line) a greater number of cells (columns) are removed from the substratum.

There were variations observed in both the number of remaining cells and the perpendicular force measurements. This may be due to different factors. Due to time constraints samples were made in duplicate and only two areas were scanned per sample. The initial cell number in the field of view was not the same for each scan area and varied from 34 cells as the lowest and 116 cells as the highest (Figure 4). If the initial cell number is high, the cells are more closely packed and have both contact with the surface and surrounding cells. That can make them more difficult to “push off” the surface. A force distance curve was made after each scan to measure and calculate the perpendicular force. It is not possible to set the perpendicular force to a specific value which means that the start, increase and end value of the perpendicular force can vary from each scanning set resulting in a high standard deviation on the mean of two repeated samples.

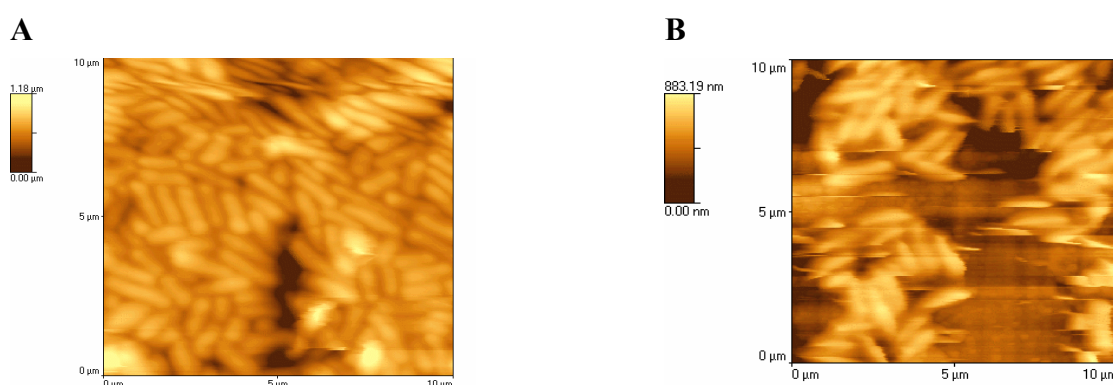


Fig 3. AFM images of *L. monocytogenes* cells spotted on stainless steel illustrating differences in high initial cell number (A) and low initial cell number (B)

When using the AFM to study removal of *S. aureus* and *P. aeruginosa*, (two very different bacteria), the results indicate a very clear difference due to cell shape and surface topography. In our experiment, we compared the adhesion strength of two different strains of the same species. If there is a difference this would be expected to be very small, since it will depend on the biochemistry (and therefore surface interactions at the nano scale) of the microbial cell surface rather than the cell shape (which varied in surface area in micron scale). Therefore, scanning of the cells with the AFM-tip may require numerous replications to enable the discrimination of adhesion forces on this scale.

It was not possible to study non-washed cells of N53-1 and Scott A since the organic material covers the cells. Hence, the washing step may mask any NaCl-induced change on the cell.

No difference was found in length and width of cells due to strain or cultivation conditions.

Conclusion

Scott A appeared to adhere more tightly when grown with NaCl as compared to cells grown without NaCl. This difference was not observed for N53-1. No difference in adhesion strength was seen between strains. To determine any strain differences or further study on influence of NaCl on adhesion it is necessary to scan more samples.

The AFM is a very sensitive tool, but it may not be possible to use the method to detect differences in adhesion strength for two bacteria of the same strain. However, adapted use of this method for example using a different liquid – eg. a weak solution of disinfectant, or a salt solution may result in greater differences seen in the strength of adhesion between strains.

The exchange has been very useful since it has given me a good insight into the use, advantages and disadvantages of this method and it is now possible for me to understand better what this method can be used for. Also, I have obtained a lot of excellent pictures of *L. monocytogenes* cells and ideas for future work.

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Paper 1

Kastbjerg, V.G. and Gram, L.

Model systems allowing quantification of sensitivity to disinfectants and comparison of disinfectant susceptibility of persistent and presumed nonpersistent *Listeria monocytogenes*.

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ORIGINAL ARTICLE

Model systems allowing quantification of sensitivity to disinfectants and comparison of disinfectant susceptibility of persistent and presumed nonpersistent *Listeria monocytogenes*

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Keywords

attached bacteria, disinfection susceptibility, *Listeria monocytogenes*, NaCl, persistence, planktonic bacteria.

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Abstract

Aims: To determine if *Listeria monocytogenes* persistent strains differ from presumed nonpersistent strains in disinfection susceptibility and to examine the influence of attachment and NaCl on susceptibility.

Methods and Results: Two model-systems that allowed quantitative inter-strain comparison of disinfectant sensitivity were developed. Persistent *L. monocytogenes* were not more tolerant to the disinfectants Incimaxx DES and Triquart SUPER than presumed nonpersistent isolates. When calibrating the systems with respect to presence of biological material and cell density, attached bacteria were as sensitive to disinfectants as were planktonic bacteria. Growth with 5% NaCl increased the tolerance of planktonic cells to Incimaxx DES. All strains of spot inoculated *L. monocytogenes* survived well 20 h of drying when protected by growth media and 5% NaCl, but were not protected by NaCl against disinfection.

Conclusions: Persistent strains of *L. monocytogenes* are as susceptible to disinfectants as are presumed nonpersistent strains and attachment does not render the strains more tolerant to disinfectants. Growth with NaCl affected the susceptibility of the planktonic *L. monocytogenes* to Incimaxx DES and protected spot inoculated cells during drying.

Significance and Impact of the Study: Attachment to surfaces does not *per se* offer protection to *L. monocytogenes* against disinfectants and disinfection tolerances do not appear to influence the ability of a strain to persist.

Introduction

The food-borne, infectious bacterium *Listeria monocytogenes* has a remarkable ability to reside in food processing plants (Unnerstad *et al.* 1996; Miettinen *et al.* 1999; Chasseignaux *et al.* 2001; Norton *et al.* 2001; Lundén *et al.* 2003; Wulff *et al.* 2006), where it can be repeatedly isolated from the production environment. Although the bacterium is an environmental bacterium present on raw materials for food production, the immediate source of product contamination is often the processing environment itself (Rørvik *et al.* 1995; Autio *et al.* 1999; Giovannacci *et al.* 1999; Miettinen *et al.* 1999; Norton *et al.*

2001; Lundén *et al.* 2003; Wulff *et al.* 2006). Some *L. monocytogenes* strains can persist for years in food processing plants (Miettinen *et al.* 1999; Vogel *et al.* 2001a; Holah *et al.* 2002; Wulff *et al.* 2006; Keto-Timonen *et al.* 2007) with persistence being defined as the ability to repeatedly isolate a specific molecular subtype or strain in the same factory during an extended period of time; typically months or years. Hence, the persistent strains may cause repeated product contamination. We recently demonstrated that a particular random amplified polymorphic DNA (RAPD) type (RAPD type 9) was persistent in several fish processing facilities (Wulff *et al.* 2006) and had been detected in fish processing for more than 8 years

(Vogel *et al.* 2001b; Wulff *et al.* 2006), although this RAPD type is not common in the outside environment (Hansen *et al.* 2006).

Listeria monocytogenes can cause invasive listeriosis in immunocompromised individuals, infants and the elderly. The disease is relatively rare; 3·4 cases per million inhabitants in Europe (Valk *et al.* 2005), but with a high fatality rate (25–30%) (FAO/WHO, 2004). The number of cases is currently on the increase in several European countries (EFSA, 2007), however, the reasons for this are not known and a potential link to long-term persistent subtypes should be investigated. Understanding the genetic and physiological factors determining the persistence of certain subtypes of *L. monocytogenes* would be crucial in reducing contamination.

One could hypothesize that some *L. monocytogenes* persist because of their ability to adhere to surfaces. Whilst some studies have concluded that persistent strains of *L. monocytogenes* adhere better to surfaces than nonpersistent strains (Norwood and Gilmour 1999; Lundén *et al.* 2000; Borucki *et al.* 2003), others have found no relationship between environmental persistence of strains and ability to adhere (Djordjevic *et al.* 2002; Jensen *et al.* 2007). Environmental factors such as NaCl may enhance autoaggregation and adhesion of *L. monocytogenes* (Jensen *et al.* 2007); however, this is not unique to persistent strains. It has also been proposed that persistent strains are better to sustain different food stresses such as low temperature, NaCl, acid and heat. However, no systematic pattern has emerged when comparing growth and stress tolerance of persistent and presumed nonpersistent strains (Jensen *et al.* 2007; Lundén *et al.* 2008; Porsby *et al.* 2008). Together, the studies do not point out a clear attribute that could explain the persistence of these specific subtypes of *L. monocytogenes*.

It has been suggested that persistent strains could persist because of resistance to cleaning and disinfection agents. Such resistance could be a result of long time exposure to subinhibitory concentrations (To *et al.* 2002). Resistance is a relative term and often, strains are categorized as resistant when displaying a higher minimal inhibitory concentration (MIC). The term is easily understood in the area of antibiotics, as they are used at levels of MIC, however, disinfectants are used at concentrations much higher than the MIC-value and therefore decreased susceptibility of a bacterial strain will not necessarily lead to higher survival after disinfection. We therefore use the term 'tolerant' as defined by Lewis (2007) as 'the ability of cells to survive killing by antimicrobial without expressing or using resistant mechanisms'. Aase *et al.* (2000) found that 10% of *L. monocytogenes* strains isolated from food processing production environments, raw products, and finished products had increased

tolerance to benzalkonium chloride. Strains with enhanced benzalkonium chloride tolerance and from the Norwegian fish processing plant were all persistent, indicating that such increased tolerance could be a factor correlated to persistence. However, other studies have not been able to demonstrate a correlation between persistence and enhanced tolerance to disinfectants (Earnshaw and Lawrence 1998; Holah *et al.* 2002; Heir *et al.* 2004). The studies mentioned have all studied planktonic bacteria and one could hypothesize that the tolerance pattern of bacteria attached to surfaces would be different.

Higher concentrations of disinfectant appear to be needed to eliminate attached cells of *L. monocytogenes* as compared with planktonic cells (Frank and Koffi 1990; Norwood and Gilmour 2000; Stopforth *et al.* 2002; Robbins *et al.* 2005; Folsom and Frank 2006). However, Stopforth *et al.* (2002) found, that detached cells were as sensitive as planktonic cells to the disinfectants. This could indicate that the higher concentration needed for the elimination of attached bacteria than for bacteria in suspension is more likely a result of bacterial shielding than to a change in sensitivity of the attached cell *per se*. In the present study, we have compared sensitivity of planktonic and attached bacteria in a set up that allows calibration to the same biomass and biological material allowing us to clarify this issue.

The purpose of this study was to determine if strains of *L. monocytogenes* that persist in the fish processing environment differ from other presumed nonpersistent strains in their susceptibility to well-known disinfectants. We also address the influence of physiological state of the bacterium (attached/planktonic) and of a common food preservative (NaCl) that causes *L. monocytogenes* to autoaggregate and adhere strongly to plastic surfaces (Jensen *et al.* 2007).

Materials and methods

Bacterial strains and media

Fourteen strains of *L. monocytogenes* (Table 1) representing different serotypes, RAPD types, and origins (food processing, environment, human, animal and food) were used. Five of the strains represent RAPD types 9, 12 and 15 (Vogel *et al.* 2001a,b; Wulff *et al.* 2006) that have been or are still persistent in several fish-processing industries. Three strains including the type strains Scott A and LO28 were human isolates belonging to the different RAPD types. Two strains were isolated from food and one of these 7291 belongs to RAPD type 15, the same as one of the persistent strains. Strain 7418 represents an RAPD type often isolated from foods (Vogel *et al.* 2004). The type isolate, EGD and a sigB mutant of this, 2315

Table 1 Origin, RAPD type, serotype and lineage of strains of *Listeria monocytogenes* used in the present study

Strain	Origin	Frequently occurring, persistent RAPD type in processing plants	RAPD type	Serotype	Lineage	Reference
N53-1	Smoke house equipment	+	9	1/2a	2	Wulff <i>et al.</i> 2006
La111	Cold-smoked salmon	+	9	1/2a	2	Vogel <i>et al.</i> 2001a
La22	Cold-smoked salmon	+	12	1/2a	2	Vogel <i>et al.</i> 2001b
V518a	Smoke house equipment	+	15	4b	1	Vogel <i>et al.</i> 2001b
7291	Pasta with chicken	+	15	4b	1	Larsen <i>et al.</i> 2002
3R1	Raw salmon	–	5	ND	ND	Wulff <i>et al.</i> 2006
7418	Spreadable sausage	–	14	1/2b	1	Larsen <i>et al.</i> 2002
N22-2	Floor in smoke house	–	48	ND	ND	Wulff <i>et al.</i> 2006
EGD	Rabbit 1926	–	68	1/2a	2	W. Goebel*
2315	EGD sigB deletions mutant					Brøndsted <i>et al.</i> 2003
LO28	Human, faecal	–	69	1/2c	2	Vázquez-Boland <i>et al.</i> 1992
4446	Human, clinical	–	71	1/2b	1	Larsen <i>et al.</i> 2002
Scott A	Human, clinical	–	72	4b	1	M. Doyle†
Br22	Sediment in fresh water fish tank	–	?‡	1/2a	2	Hansen <i>et al.</i> 2006

ND, not determined; RAPD, random amplified polymorphic DNA.

*The strain was kindly provided by Werner Goebel, University of Würzburg.

†The strain was kindly provided by Mike Doyle, University of Georgia, USA.

‡The RAPD type is different from the persistent RAPD types from processing plants.

(Brøndsted *et al.* 2003) were included. Br22 is a strain frequently isolated from the environment (Hansen *et al.* 2006) representing a RAPD type different from the persistent. Strains 3R1 and N22-2 were isolated from the fish processing industry and represent RAPD types 5 and 48, respectively, only found once. These strains we do denote as presumed nonpersistent because they could in principle be persistent in other settings. The 14 strains were obtained from DTU Aqua (formerly The Danish Institute for Fishery Research), Faculty of LIFE science (formerly The Royal Veterinary and Agricultural University), the FOOD Institute, DTU (formerly The Danish Institute for Food and Veterinary Research), Statens Serum Institute, University of Würzburg, Germany and University of Georgia, USA. Stock cultures were stored at -80°C in 4% (w/v) glycerol. The bacteria were cultivated on brain heart infusion (BHI) agar (CM0225; Oxoid, supplemented with 1.5% agar) for 1–3 days at 30°C . Subsequent cultures were prepared in tryptone soya broth (TSB; CM129, Oxoid) supplemented with glucose to a final concentration of 1% (w/v) plus in some trials addition of NaCl to a final concentration of 5% (w/w). The bacteria were grown overnight at 30°C , diluted to a factor of 1000 and grown overnight at 30°C .

Preparation of stainless steel coupons for spot inoculation and attachment studies

Stainless steel coupons (AISI 316, unpolished, 2B finish) cut into 10- by 20-mm with a thickness of 1 mm were soaked in 15% Deconex solution (15 PF; Borer Chemie

AG, Zuchwill, Switzerland) for 60 min, autoclaved and rinsed with demineralized water. The stainless steel coupons were air dried and soaked in acetone for 60 min. After air drying, the coupons were sterilized by autoclaving before use.

Evaluation of set ups for surface attached bacteria

To quantify the differences in strain sensitivity to disinfectants, a 'window' of reduction in bacterial numbers must be obtained. By this we mean that there is a quantifiable reduction, i.e. that some bacterial cells remain after the exposure to disinfection. This allows us to compare real log reductions and compare strains. This requires that the initial number of bacterial cells on the surface is relatively high. To obtain this, different set ups were evaluated and optimized. First, *L. monocytogenes* was allowed to grow and adhere in a vertical and horizontal batch system. The vertical set up was modified after Bagge *et al.* (2001). Briefly, stainless steel coupons were clamped vertically in a steel circular rack placed in a beaker with 250 ml TSB with 1% glucose. The horizontal system consisted of stainless steel coupons placed horizontal in a Petri dish (14 cm diameter) with 100 ml TSB with 1% glucose. The beaker and Petri dish were inoculated with *L. monocytogenes* N53-1, precultured in TSB with 1% glucose, to a final concentration of approx. $3 \log_{10}$ CFU ml^{-1} and incubated at 25°C . Bacterial density in the solutions was determined in a similar manner and at the same time points (24, 48, 72, 96 h) as coupons were sampled for measurements of attached bacteria. When

sampling, each coupon was rinsed with 5 ml of sterile phosphate-buffered saline [0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.144% (w/v) Na₂HPO₄, 0.024% (w/v) NaH₂PO₄, pH 7.4] and dabbed on adsorbent paper to remove nonattached or loosely attached bacteria before quantification of attached bacteria. To optimize further on the number of attached bacteria in the horizontal set up, bacterial cells were allowed to attach at different temperatures (15, 20, 25, 37°C) and for increased time in TSB with 1% glucose with and without 5% NaCl. In addition, at 20°C, growth media were changed at 2 and 24 h, and the cell numbers were quantified at 24 and 48 h (Chavant *et al.* 2004). Furthermore, we tested a set up where bacterial cells were spot inoculated by a method modified after Kim *et al.* (2007). Sterile stainless steel coupons were placed on a wire screen in a laminar-flow biosafety cabinet. Of an outgrown culture of the strains tested, 1 ml was centrifuged at 3000 g for 10 min. Pellet was re-suspended in 100 µl sterile growth media, thereby increasing the cell-number, and 20 µl was deposited on each coupon to give a count above 8 log₁₀ CFU per coupon (Table 5). The inoculum was dried for 20 h in the laminar-flow biosafety cabinet at 25°C.

Quantification of attached bacteria

Bacteria were detached from coupons by sonication for 4 min (28 kHz, 2 × 150 W sonication bath, Delta 220; Deltasonic, Meaux, France) (Leriche and Carpentier 1995) in 3 ml sterile peptone saline, vortexed at maximum speed for 15 s and diluted 10-fold serially. Colony counts were enumerated on BHI agar. Colony counts on samples with and without sonication were identical, indicating that the sonication *per se* had no adverse effect on the bacteria. The sonication treatment removed 99% of the cells from the SS coupons.

Preparation of disinfectant solutions

Two disinfectants commonly used in the food industry were evaluated in this study. Incimaxx DES (Ecolab Denmark ApS, Valby, Denmark) is used both as disinfectant and for decalcification. The active ingredients are peroxy acids and hydrogen peroxide. Triquart SUPER (Ecolab Denmark ApS) is used for disinfection at open surfaces daily in the food industry. The active ingredient was a quaternary ammonium compound. Disinfectants were diluted in sterilized, demineralized water to obtain concentrations where evaluation of differences between strains was possible. Disinfectant solutions were prepared 10 times the strength of desired treatment right before use for testing planktonic cells and in desired concentrations for disinfection of spot inoculated cells.

Disinfection of *Listeria monocytogenes* as planktonic cells

One millilitre of an outgrown culture was centrifuged as described earlier and the pellet was re-suspended in 0.9% (w/v) NaCl. To standardize the biomass, the solution was adjusted in 0.9% NaCl to an optical density at 450 nm (OD₄₅₀) of 0.120–0.129. Forty microlitres of a tempered disinfection solution was mixed with 360 µl bacterial suspension in Eppendorf tubes (1.5 ml) and placed in a water bath at 20 ± 1°C. Sterile demineralized water was used as control. After 20 min, 200 µl Dey-Engly (DE) neutralizing broth (281910; Difco, BD, Sparks, France) was added. Preliminary experiments showed that DE broth neutralized disinfectants also in higher concentrations of disinfectants than used and that viability of *L. monocytogenes* was unaffected when cells were suspended in DE broth. Bacterial numbers were determined by surface plating on BHI agar plates that were incubated at 37°C for 2 days. Each concentration of disinfectant was tested in triplicate and the complete experiment was repeated at least twice. Based on a set of preliminary experiments of sensitivity, concentrations of 0.0062%, 0.0031% and 0.0015% (v/v) were used for Incimaxx DES and 0.04%, 0.02% and 0.01% (v/v) for Triquart SUPER.

The effect of presence of sodium chloride on disinfection susceptibility

To determine if differences seen in disinfection susceptibility to Incimaxx DES of planktonic bacterial cells grown with and without 5% NaCl was due to the mere presence of NaCl, 1 ml of outgrown cultures of *L. monocytogenes* N53-1 and N22-2 grown with and without NaCl was centrifuged as described earlier, and the pellet was re-suspended in 0.9% (w/v) or 5% (w/v) NaCl. The amount of biomass was standardized and the bacterial cells were disinfected as described earlier.

Disinfection of *Listeria monocytogenes* as attached cells

Spot inoculated coupons were placed horizontally in a Petri dish and 150 µl of disinfectant was distributed on the surface. Sterilized demineralized water was used as control. After 20 min of incubation, the coupons were transferred with the disinfectant to 3 ml DE neutralizing broth and sonicated as described earlier. Also, three coupons denoted as untreated were transferred directly to DE neutralizer broth and sonicated. Cell numbers were determined by surface plating on BHI agar plates that were incubated at 37°C for 2 days. Each concentration of disinfectant was tested in triple determinations, and the experiment was repeated at least twice. For Incimaxx DES, concentrations of 0.125%, 0.062% and 0.031% (v/v)

were tested. For Triquart SUPER, the tested concentrations were 0.16%, 0.08% and 0.04% (v/v).

Evaluation of the presence of biological material on disinfection susceptibility

A higher concentration of disinfectant was needed to kill spot inoculated dried bacteria than planktonic bacteria. To determine if this was due to the presence of a higher biological load (i.e. more bacteria and growth media), we prepared suspended bacteria in a completely parallel manner to spot inoculated bacteria (e.g. 10 times more bacteria suspended in growth media) and exposed them to exactly the same volume and concentration of disinfectant. One millilitre of an outgrown culture of N53-1 in TSB with 1% glucose was centrifuged and suspended in 100 μ l sterile media. A 150 μ l of a tempered disinfection solution was mixed with 20 μ l of bacterial suspension in Eppendorf tubes (1.5 ml) and placed in a water bath at $20 \pm 1^\circ\text{C}$. Sterile demineralized water was used as control. After 20 min, 830 μ l DE neutralizing broth was added. The mixture was transferred to 2 ml DE neutralizer broth to attain a total volume of 3 ml and sonicated for 4 min. Furthermore, of the same inoculum, 20 μ l was spotted on stainless steel coupons, dried and disinfected as described earlier. Bacterial numbers were determined by surface plating on BHI agar plates that were incubated at 37°C for 2 days. Concentrations of 0.125%, 0.062% and 0.031% Incimaxx DES were used. Each concentration of disinfectant was tested in triplicate. To obtain the same initial cell number before disinfection, the experiment was also carried out with planktonic cells resuspended in 1 ml of sterile media, whereas cell-culture for spot inoculation still was up-concentrated.

Statistical analysis

All counts were log-transformed. Counts of zeros were regarded as 1 and log-transformed. We used a two-way ANOVA followed by Bonferroni post-test to statistically compare the number of attached bacteria as influenced by growth media and positions of coupons. We used a Student's *t*-test with pretesting of equal variances to determine if the level of attached bacteria as influenced by different growth temperatures or growth media was statistically different. For disinfection trials with planktonic cells, statistical comparisons were made between \log_{10} reductions calculated as \log_{10} reduction = \log_{10} surviving cells after water treatment (control) – \log_{10} surviving cells after disinfection treatment. Two means were statistically compared using Student's *t*-test with a significance level of $P < 0.05$, whereas one-way ANOVA was used for analysing three or more means followed by Tukeys post-test.

Statistically significant differences in the remaining bacteria after disinfection due to presence of different amounts of NaCl during growth and disinfection were analysed by two-way ANOVA. Statistically significant differences in surviving cells after drying due to differences in the NaCl were found comparing means by Student's *t*-test with pre-testing of equal variances.

Results

Evaluation of set ups for surface attached bacteria

As disinfectants aim at reducing the surface count, comparison of strain sensitivity requires that some bacteria are left on the surface after disinfection. Hence, our study required a model system with a high, initial bacterial number on the surface. *L. monocytogenes* N53-1 attached readily to coupons placed both horizontally and vertically with a higher number on the former ($5.98 \pm 0.25 \log_{10}$ CFU coupon $^{-1}$) than on the latter ($5.37 \pm 0.1 \log_{10}$ CFU coupon $^{-1}$) after 24 h ($P < 0.05$) (Table 2). This significant difference was seen also at 48 h ($P < 0.05$) and 72 h ($P < 0.01$) but not at 96 h ($P > 0.05$). There was no difference in CFU ml $^{-1}$ in the two suspensions ($P = 0.09$) (data not shown).

Attempts were made to increase the number of attached bacteria in the horizontal set up by using different

Table 2 Number of attached cells of *Listeria monocytogenes* N53-1 on stainless steel coupons after surface attachment or spot inoculation in TSB with 1% glucose with or without 5% NaCl in different model set ups

Model	Temperature ($^\circ\text{C}$)	Time (h)	Log $_{10}$ (CFU coupon $^{-1}$) \pm SD*	
			–NaCl	+NaCl
Vertical	25	24	5.37 ± 0.1	ND
		48	5.48 ± 0.3	ND
Horizontal	25	24†	5.98 ± 0.25	ND
		48†	6.08 ± 0.19	ND
		49‡	5.82 ± 0.32	7.07 ± 0.03
		49	7.44 ± 0.10	6.45 ± 0.18
Horizontal with renewal§	20	24	6.44 ± 0.36	6.18 ± 0.43
		48	7.22 ± 0.06	6.48 ± 0.17
Spot inoculated and dried	25	0	8.04 ± 0.06	8.01 ± 0.01
		0.5	7.87 ± 0.08	7.99 ± 0.05
		20	7.05 ± 0.12	7.84 ± 0.03

ND, not determined.

*Numbers are the average of triple determinations. SD is standard deviation from the triplicate measurements.

†Experiment 1.

‡Experiment 2.

§Growth medium was renewed at 2 and 24 h.

incubation temperatures and times. Also, the effect of growth with NaCl on attachment of cells on an inert surface was studied. At 15 and 20°C, the highest number of bacteria attached to the stainless steel coupons were between 6 log₁₀ and 7 log₁₀ CFU coupon⁻¹ being reached after 73–96 h (data not shown). This level was obtained at 49 h at 25°C, whereas levels slightly higher than 7 log₁₀ CFU coupon⁻¹ were obtained at 37°C (Table 2). NaCl appeared to increase the number of bacteria attaching to the surfaces at 20°C and significantly at 25°C ($P < 0.05$), but not at 15 and 37°C (data not shown, Table 2). *Listeria monocytogenes* N53-1 grew to and stabilized at 8 log₁₀ in the suspensions at all temperatures both with and without NaCl, and differences in suspension cell density are therefore not causing the different numbers attaching to the surface. At 37°C, the CFU ml⁻¹ decreased to 6 log₁₀ for TSB with 1% glucose from 24 to 49 h (data not shown). However, the decrease to 4 log₁₀ in attached bacterial cells from 24 to 49 h must also reflect detachment of the bacterial cells.

Renewal of growth media after 2 and 24 h caused a slight further increase of attached *L. monocytogenes* N53-1 at 20°C (Table 2). However, this was not higher as compared to the number of attached cells when grown at 37°C.

Preliminary experiments with disinfection of the surface attached bacteria from a horizontal set up were carried out and revealed that just soaking in water reduced counts with 2–3 log units. Therefore, a set up giving a high bacterial count with possibility of horizontal treatment was required. A CFU coupon⁻¹ of c. 8 log₁₀ was obtained when using the set up with spot-inoculated dried bacterial cells modified after Kim *et al.* (2007). Treatment with water, when the coupon was placed horizontal, caused only a 0.5–1 log₁₀ reduction. Therefore, this method was preferred because of the high initial cell-number, which could be standardized. As cells were only spotted on one side of the coupons, they could be disinfected horizontally, thereby imitating the industrial process.

Disinfection of strains with Incimaxx DES

We hypothesized that strains could be persistent because of decreased susceptibility to disinfectants used in the food industry and exposed strains to a commercial disinfectant both as planktonic and attached (spot inoculated, dried) bacteria. The CFU ml⁻¹ and CFU coupon⁻¹ after disinfection were lowered as expected with increasing concentration of Incimaxx DES for all strains. At 0.0062%, a dramatic reduction of all planktonic strains was seen, while at 0.0015% they were almost unaffected (data not shown). Disinfecting with 0.0031% Incimaxx DES caused a 2–5 log₁₀ reduction and allowed inter-strain

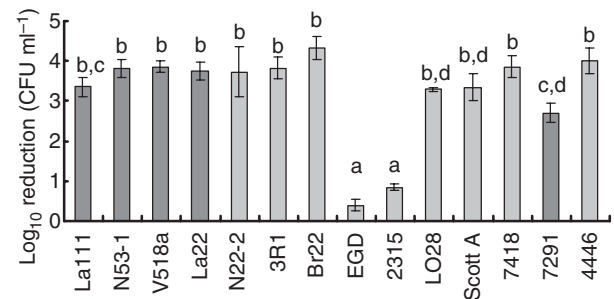


Figure 1 Reduction in cell-number of *Listeria monocytogenes* strains after 20 min disinfection with 0.0031% Incimaxx. Persistent strains are in dark grey and other presumed nonpersistent in light grey. Columns are average from one trial carried out in triplicate. Error bars are based on the standard deviation from the triplicate measurements. The results are representative of two independent experiments. For comparison of the differences in reduction between strains, columns that are not denoted by the same letter (a, b, c, d) are significantly different ($P < 0.05$).

comparisons of sensitivity differences. A few strains (EGD and its sigB mutant, 2315) were more tolerant than the majority ($P < 0.05$) (Fig. 1); however, strains belonging to the persistent RAPD types (La111, N53-1, V518a, La22, 7291) were as susceptible to Incimaxx DES, as planktonic bacteria, as compared with the remaining strains.

Six strains (N53-1, V518a, N22-2, Scott A, 7418 and 4446) were disinfected as spot inoculated dried bacterial cells. All strains were killed at 0.125% Incimaxx DES whereas they were almost unaffected by 0.031%. Disinfection with 0.062% Incimaxx DES allowed inter-strain comparisons. Minor differences in sensitivity were found, but the persistent RAPD types (N53-1, V518a) were not more tolerant as compared with the other strains (Fig. 2).

Incimaxx DES disinfection of planktonic *Listeria monocytogenes* grown with or without sodium chloride

Growth with 5% NaCl changes the adhesion pattern of the *L. monocytogenes* (Table 2) and causes the bacteria to aggregate (Jensen *et al.* 2007), and we hypothesized that NaCl could affect the sensitivity of *L. monocytogenes* to disinfectants. Fourteen strains were grown in TSB with 1% glucose and TSB with 1% glucose and 5% NaCl and disinfected with three concentrations of Incimaxx DES. Growth with 5% NaCl enhanced the tolerance of several *L. monocytogenes* to 0.0031% Incimaxx (Table 3). The difference in reduction was statistically significant for seven strains (La111, N53-1, N22-2, 3R1, Br22, Scott A and 7291) in both experiments ($P < 0.05$). For six strains (V518a, La22, 2315, LO28, 7418 and 4446), the reduction

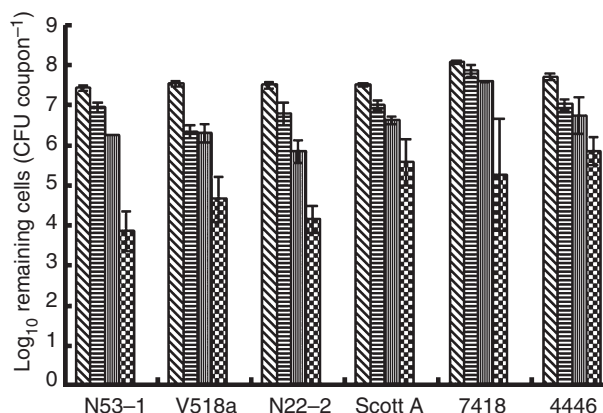


Figure 2 Remaining cell-number of *Listeria monocytogenes* strains on stainless steel after 20 h drying (■) and 20 min treatment with water (□), 0.031% (▨), 0.062% (▩) and 0.125% (▧) Incimaxx DES. Persistent strains are N53-1 and V518a. Columns are average from one trial carried out in triplicate. Error bars are based on the standard deviation from the triplicate measurements. The results are representative of two independent experiments.

in bacterial number was significantly higher for the strains grown in TSB with 1% glucose as compared with the strain grown with 5% NaCl in one of the experiments ($P < 0.05$), while the difference was not significant in the other experiment ($P > 0.05$). Strain EGD was reduced more markedly by Incimaxx DES when grown without NaCl ($P < 0.05$).

The effect of presence of sodium chloride on disinfection susceptibility

To determine if the difference in tolerance to Incimaxx DES was due to the presence of extra NaCl on the cell-surfaces on the cells grown with NaCl, the strain N53-1 and N22-2 were grown in TSB with 1% glucose with or without 5% NaCl, dissolved in 0.9% NaCl or 5% NaCl and disinfected with 0.0031% Incimaxx. The reduction in bacterial numbers was independent of concentration of NaCl in the solvent (Table 4), however, the survival was dependent on pregrowth media for both strains ($P < 0.001$). The data indicate that the growth with NaCl enhance the tolerance of the bacterial cells to Incimaxx DES, but this enhanced tolerance is not due to the presence of a higher concentration of NaCl during the disinfection.

Incimaxx DES disinfection of spot inoculated and dried *Listeria monocytogenes* grown with or without sodium chloride

Six strains (N53-1, V518a, N22-2, Scott A, 7418 and 4446) were disinfected with Incimaxx DES as spot inoculated, dried bacterial cells. The initial number of

bacteria applied to the surfaces was identical for strains cultured with and without 5% NaCl. However, after 20 h of drying, the CFU coupon⁻¹ were significantly higher for cells grown and dried in TSB with 1% glucose and 5% NaCl as compared with cells grown and dried in TSB with 1% glucose, indicating that bacteria dried in media with NaCl were better protected during the 20 h of drying (Table 5). This difference in protective effect of growth media was not as clear after short drying of 30 min, as after the longer drying of 20 h. However, when disinfecting the strains with 0.062% Incimaxx DES, bacteria dried with 5% NaCl were more sensitive than or as sensitive as bacteria dried in media without 5% NaCl indicating that the presence and protection from NaCl under drying do not protect the cells under disinfection (Table 6).

Triquart SUPER disinfection of planktonic *Listeria monocytogenes* grown with or without sodium chloride

We also evaluated the disinfection sensitivity of *L. monocytogenes* to Triquart SUPER, which contains a quaternary ammonium compound. Six strains (N53-1, V518a, N22-2, Scott A, 7418 and 4446) were grown in TSB with 1% glucose and TSB with 1% glucose and 5% NaCl and disinfected with three concentrations of Triquart SUPER to study if there were differences between strains, and if growth with NaCl had an influence on disinfectant sensitivity as seen for Incimaxx DES. The reduction measured as CFU ml⁻¹ after disinfection was increased as expected with increased concentration of Triquart SUPER. When using concentrations of 0.04% and 0.01% Triquart SUPER, there were no differences between strains. With disinfection by 0.04% Triquart SUPER, all strains were strongly affected while at 0.01% no change in bacterial numbers was seen compared to treatment with water. At a concentration of 0.02% Triquart SUPER, inter-strain comparisons could be done. The persistent RAPD types represented by N53-1 and V518a were not more tolerant to Triquart SUPER as compared with the remaining four strains. Furthermore, growth with 5% NaCl did not enhance disinfection sensitivity, because bacteria grown with 5% NaCl were more sensitive than or as sensitive as bacteria grown in media without supplementation with NaCl (Table 3).

Triquart SUPER disinfection of spot inoculated and dried *Listeria monocytogenes* grown with or without sodium chloride

The six strains (N53-1, V518a, N22-2, Scott A, 7418 and 4446) were grown, spot inoculated and dried in TSB with a total of 1% glucose with or without a total of 5% NaCl

Table 3 Cell number of planktonic *Listeria monocytogenes* strains after 20 min treatment with water (control) and reduction in cell number after 20 min disinfection with Incimaxx DES or Triquart SUPER

Strain	No.	0.0031% Incimaxx DES				0.02% Triquart SUPER			
		Log ₁₀ control (CFU ml ⁻¹) ± SD*		Log ₁₀ reduction (CFU ml ⁻¹) ± SD*†		Log ₁₀ control (CFU ml ⁻¹) ± SD*		Log ₁₀ reduction (CFU ml ⁻¹) ± SD*†	
		-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl	-NaCl	+NaCl
La111	1	7.81 ± 0.03	7.84 ± 0.07	2.40 ± 0.18	1.37 ± 0.19A‡	ND	ND	ND	ND
	2	7.80 ± 0.1	7.89 ± 0	2.51 ± 0.10	1.90 ± 0.08A	ND	ND	ND	ND
N53-1	1	7.73 ± 0	7.41 ± 0.13	2.31 ± 0.10	1.03 ± 0.04A	7.83 ± 0.08	8.25 ± 0.01B	2.90 ± 0.18	2.92 ± 0.23
	2	7.79 ± 0.07	7.41 ± 0.11B§	3.12 ± 0.01	1.47 ± 0.10A	7.89 ± 0.04	8.15 ± 0.05B	1.65 ± 0.13	1.52 ± 0.16
V518a	1	7.88 ± 0.01	7.90 ± 0.09	1.77 ± 0.17	1.30 ± 0.41	7.77 ± 0.04	8.25 ± 0.06B	0.91 ± 0.26	1.09 ± 0.14
	2	7.83 ± 0.03	7.87 ± 0.09	2.94 ± 0.07	2.07 ± 0.12A	7.88 ± 0.04	7.94 ± 0.07	0.57 ± 0.1A	1.82 ± 0.14
La22	1	7.76 ± 0.05	7.85 ± 0.01	2.56 ± 0.08	1.92 ± 0.19A	ND	ND	ND	ND
	2	7.73 ± 0.05	7.70 ± 0.06	1.89 ± 0.17	1.43 ± 0.27	ND	ND	ND	ND
N22-2	1	7.80 ± 0.04	7.78 ± 0.04	2.16 ± 0.19	0.73 ± 0.06A	7.77 ± 0.07	7.94 ± 0.1	1.08 ± 0.18A	2.96 ± 0.27
	2	7.83 ± 0.07	7.91 ± 0.05	3.11 ± 0.08	1.52 ± 0.27A	7.63 ± 0.1	8.05 ± 0.04B	0.42 ± 0.06A	1.58 ± 0.2
3R1	1	7.78 ± 0.03	7.82 ± 0.04	1.48 ± 0.59	0.43 ± 0.12A	ND	ND	ND	ND
	2	7.5 ± 0.07	7.89 ± 0.13B	3.36 ± 0.14	1.08 ± 0.12A	ND	ND	ND	ND
Br22	1	7.74 ± 0.04	7.74 ± 0.05	1.80 ± 0.05	1.30 ± 0.06A	ND	ND	ND	ND
	2	7.82 ± 0.04	7.73 ± 0.1	2.66 ± 0.45	1.65 ± 0.07A	ND	ND	ND	ND
EGD	1	6.22 ± 0.07	7.44 ± 0.07B	1.25 ± 0.12A	1.58 ± 0.10	ND	ND	ND	ND
	2	6.65 ± 0.04	7.45 ± 0.09B	0.58 ± 0.02A	1.74 ± 0.11	ND	ND	ND	ND
2315	1	6.75 ± 0.04	6.91 ± 0.04B	0.33 ± 0.04	0.33 ± 0.04	ND	ND	ND	ND
	2	6.21 ± 0.09	6.33 ± 0.04	0.39 ± 0.01	0.31 ± 0.03A	ND	ND	ND	ND
Lo28	1	7.47 ± 0.12	7.90 ± 0.06	1.77 ± 0.19	1.82 ± 0.05	ND	ND	ND	ND
	2	7.31 ± 0.01	7.77 ± 0.04B	2.72 ± 0.01	1.84 ± 0.04A	ND	ND	ND	ND
Scott A	1	7.92 ± 0.05	7.89 ± 0.08	2.56 ± 0.05	1.90 ± 0.16A	7.80 ± 0.08	7.94 ± 0.02B	1.62 ± 0.2	1.75 ± 0.09
	2	7.75 ± 0.11	7.79 ± 0.02	1.79 ± 0.26	0.28 ± 0.09A	7.71 ± 0.07	7.91 ± 0.07B	0.87 ± 0.32	1.07 ± 0.05
7418	1	7.97 ± 0.07	7.92 ± 0.02	1.17 ± 0.29	1.39 ± 0.05	7.99 ± 0.03	8.03 ± 0.06	0.5 ± 0.20	0.93 ± 0.03
	2	7.94 ± 0.09	7.84 ± 0.02	1.87 ± 0.08	1.62 ± 0.07A	7.98 ± 0.06	8.25 ± 0.02B	0.80 ± 0.19	1.09 ± 0.08
7291	1	8.02 ± 0.08	8.09 ± 0.04	2.55 ± 0.04	2.06 ± 0.05A	ND	ND	ND	ND
	2	7.78 ± 0.06	7.87 ± 0.07	2.75 ± 0.33	1.90 ± 0.10A	ND	ND	ND	ND
4446	1	7.84 ± 0.02	8.0 ± 0.04B	0.87 ± 0.06	0.53 ± 0.02A	7.91 ± 0.02	8.21 ± 0.07B	1.47 ± 0.16	1.63 ± 0.09
	2	7.85 ± 0.01	7.93 ± 0.02B	1.82 ± 0.20	2.10 ± 0.13	7.88 ± 0.04	8.37 ± 0.03B	1.04 ± 0.09	1.09 ± 0.06

ND, not determined.

*Numbers are average from one trial carried out in triplicate. Errors are based on the standard deviation from the triplicate measurements. Two trials have been carried out independently.

†Log₁₀ reduction (CFU ml⁻¹) is calculated as log₁₀ surviving cells after water treatment (control) – log₁₀ surviving cells after disinfection treatment.‡For comparison of the effect of growth with NaCl on disinfection survival, mean reductions for one strain in one trial that is followed by A is significantly lower ($P < 0.05$).§For comparison of differences in cell numbers in control trials, mean values for one strain in one trial that is followed by B is significantly different ($P < 0.05$).

to study the effect of Triquart SUPER on spot inoculated, dried bacterial cells. The CFU coupon⁻¹ was higher for bacteria dried in TSB with 5% NaCl as compared with bacteria dried in TSB with 1% glucose. When disinfecting the strains with 0.08% Triquart SUPER, bacteria dried with 5% NaCl were more sensitive than or as sensitive as bacteria dried in media without 5% NaCl indicating that the presence and protection from NaCl under drying do not protect the cells during disinfection (Table 6). The persistent strains were as susceptible to 0.08% or other concentrations of Triquart SUPER as the remaining strains.

Influence of organic material and bacterial density on disinfection tolerance

Incimaxx DES was used for inter-strain comparisons in the concentrations of 0.0031% and 0.062% for planktonic and spot inoculated bacteria, respectively, whereas Triquart SUPER was used at 0.02% and 0.08%, for planktonic and spot inoculated bacteria, respectively. The higher concentration needed for disinfection of spot inoculated, dried bacteria could be due to the changes in sensitivity of the individual bacterial cell or to the differences in the set up, as the spot inoculated bacteria *per se* carried a

Table 4 Remaining cell-number of planktonic *Listeria monocytogenes* strains grown and suspended in different amounts of NaCl after 20 min of treatment with water or 0.0031% Incimaxx

Strain	No.	Suspended	Log ₁₀ (CFU ml ⁻¹) remaining cell after 20 min treatment ± SD*			
			-NaCl†		+NaCl	
			Water (control)	0.0031% Incimaxx	Water (control)	0.0031% Incimaxx
N53-1	1	0.9% NaCl	7.75 ± 0.08	3.71 ± 0.24	7.81 ± 0.03	5.90 ± 0.02
		5% NaCl	7.63 ± 0.04	3.34 ± 1.10	7.77 ± 0.03	7.30 ± 0.05
	2	0.9% NaCl	7.71 ± 0.06	3.96 ± 0.39	7.79 ± 0.06	7.10 ± 0.13
		5% NaCl	7.75 ± 0.08	3.16 ± 0.12	7.73 ± 0.02	5.96 ± 0.07
N22-2	1	0.9% NaCl	7.78 ± 0.11	4.13 ± 0.67	7.74 ± 0.09	5.84 ± 0.08
		5% NaCl	7.75 ± 0.04	4.11 ± 0.05	7.78 ± 0.10	5.34 ± 0.15

*Numbers are the average of triple determinations. SD is standard deviation from the triplicate measurements.

†Strains were pregrown in TSB with 1% glucose with or without 5% NaCl and suspended in 0.9% or 5% NaCl. Two trials have been carried out independently for N53-1.

Table 5 Number of *Listeria monocytogenes* cells on stainless steel coupons after surface application, drying for 30 min and for 20 h

Strain	No.	Log ₁₀ (CFU coupon ⁻¹) ± SD*					
		-NaCl			+NaCl		
		Spotted	Dried (30 min)	Dried (20 h)	Spotted	Dried (30 min)	Dried (20 h)
N53-1	1	8.04 ± 0.06	7.87 ± 0.08	7.05 ± 0.12	8.01 ± 0.01	7.99 ± 0.05	7.84 ± 0.03A†
	2	8.32 ± 0.08	8.09 ± 0.13	6.52 ± 0.04	8.16 ± 0.01	8.16 ± 0.09	7.61 ± 0.07A
V518a	1	8.41 ± 0.05	8.04 ± 0.12	7.00 ± 0.17	8.13 ± 0.06	8.16 ± 0.13	8.00 ± 0.09A
	2	8.24 ± 0.01	8.28 ± 0.14	6.60 ± 0.07	8.34 ± 0.19	8.66 ± 0.04	8.01 ± 0.07A
N22-2	1	8.16 ± 0.01	7.65 ± 0.07	6.93 ± 0.05	8.12 ± 0.10	8.07 ± 0.08	7.77 ± 0.06A
	2	8.27 ± 0.01	8.26 ± 0.05	6.81 ± 0.13	8.15 ± 0.01	8.09 ± 0.08	7.45 ± 0.00A
Scott A	1	8.46 ± 0.06	8.28 ± 0.05	7.44 ± 0.13	8.12 ± 0.13	8.19 ± 0.04	7.86 ± 0.04A
	2	8.27 ± 0.01	ND	7.56 ± 0.08	8.12 ± 0.08	ND	7.87 ± 0.07A
7418	1	8.48 ± 0.04	8.45 ± 0.06	7.74 ± 0.04	8.29 ± 0	8.14 ± 0.04	7.97 ± 0.02A
	2	8.36 ± 0.08	8.38 ± 0.05	7.64 ± 0.08	8.29 ± 0.02	8.27 ± 0.04	8.03 ± 0.19A
4446	1	8.49 ± 0.10	8.29 ± 0.09	7.52 ± 0.15	8.39 ± 0.12	8.30 ± 0.08	8.01 ± 0.02A
	2	8.45 ± 0.04	ND	7.47 ± 0.07	8.28 ± 0.08	ND	8.10 ± 0.03A

ND, not determined.

*Numbers are the average of triple determinations. SD is standard deviation from the triplicate measurements.

†For comparison of the effect of presence of 5% NaCl during growth and drying for 20 h, mean values for one strain in one trial followed by A is significantly higher ($P < 0.05$).

higher load of biological material as biomass and sterile media. We then pretreated the planktonic bacteria in a manner paralleling the pretreatment of the spot inoculated bacteria (i.e. harvested bacteria were re-suspended in 10-fold less volume with TSB). This resulted, logically, in a higher number of CFU ml⁻¹ and after treatment with water, a higher number of planktonic bacteria than spot inoculated, dried cells were found (Fig. 3a). This was expected, as the planktonic bacterial cells were not dried. However, the number of surviving planktonic or spot inoculated bacteria was equal when disinfecting with 0.062% Incimaxx DES. When using 0.125% Incimaxx DES, the number of surviving planktonic bacteria was

higher than that of the spot inoculated bacteria. However, this could be due to the higher initial number of bacterial cells. When the planktonic cell culture was suspended in the same volume of TSB after harvesting (i.e. nonconcentrated), the same cell number was obtained for planktonic as for spot inoculated bacteria after water treatment, and no differences were found between the numbers of remaining bacterial cells as planktonic or spot inoculated cells after disinfection (Fig. 3b). These results indicate that the higher disinfectant concentration needed to inactivate spot inoculated, dried bacteria as compared with planktonic bacteria is not due to the increased tolerance of the attached bacteria *per se*.

Table 6 Number of *Listeria monocytogenes* cells on stainless steel coupons after drying for 20 h, followed by 20 min treatment with water or disinfectant

			Log ₁₀ (CFU coupon ⁻¹) ± SD*		Log ₁₀ (CFU coupon ⁻¹) ± SD*		
			Incimaxx DES		Triquart SUPER		
Strain	No.	Treatment	–NaCl†	+NaCl	Treatment	–NaCl	+NaCl
N53-1	1	Dried (20 h)	7.05 ± 0.12	7.84 ± 0.03	Dried (20 h)	6.17 ± 0.10	8.06 ± 0.07
		Water	6.43 ± 0.08	7.23 ± 0.19	Water	6.06 ± 0.14	7.95 ± 0.02
		0.062%	5.07 ± 0.2	<1.5‡	0.08%	4.76 ± 0.13	5.92 ± 0.20
	2	Dried (20 h)	6.52 ± 0.04	7.61 ± 0.07	Dried (20 h)	7.39 ± 0.06	8.17 ± 0.11
		Water	6.18 ± 0.15	7.47 ± 0.05	Water	6.75 ± 0.04	8.18 ± 0.07
		0.062%	<1.5	<1.5	0.08%	6.11 ± 0.11	6.23 ± 0.54
V518a	1	Dried (20 h)	7.00 ± 0.17	8.00 ± 0.09	Dried (20 h)	6.02 ± 0.25	8.08 ± 0.07
		Water	6.39 ± 0.13	7.63 ± 0.02	Water	5.16 ± 0.10	7.98 ± 0.02
		0.062%	5.81 ± 0.21	<1.5	0.08%	3.63 ± 0.46	5.09 ± 0.43
	2	Dried (20 h)	6.60 ± 0.07	8.01 ± 0.07	Dried (20 h)	6.98 ± 0.02	8.09 ± 0.12
		Water	6.48 ± 0.05	7.73 ± 0.05	Water	4.74 ± 0.24	7.83 ± 0.01
		0.062%	6.09 ± 0.28	7.39 ± 0.08	0.08%	2.87 ± 0.24	3.91 ± 0.88
N22-2	1	Dried (20 h)	6.93 ± 0.05	7.77 ± 0.06	Dried (20 h)	7.02 ± 0.12	8.04 ± 0.05
		Water	6.41 ± 0.10	7.56 ± 0.04	Water	6.76 ± 0.03	7.99 ± 0.03
		0.062%	1.30 ± 2.26	<1.5	0.08%	5.98 ± 0.10	4.94 ± 0.30
	2	Dried (20 h)	6.81 ± 0.13	7.45 ± 0.00	Dried (20 h)	7.50 ± 0.06	8.18 ± 0.03
		Water	6.23 ± 0.17	7.35 ± 0.07	Water	6.95 ± 0.20	8.00 ± 0.13
		0.062%	0.91 ± 1.58	<1.5	0.08%	5.77 ± 0.27	4.70 ± 0.67
Scott A	1	Dried (20 h)	7.44 ± 0.13	7.86 ± 0.04	Dried (20 h)	6.84 ± 0.10	7.98 ± 0.06
		Water	6.35 ± 0.16	7.47 ± 0.04	Water	6.59 ± 0.03	7.81 ± 0.02
		0.062%	4.34 ± 1.02	<1.5	0.08%	5.88 ± 0.08	3.01 ± 2.78
	2	Dried (20 h)	7.56 ± 0.08	7.87 ± 0.07	Dried (20 h)	6.83 ± 0.11	7.91 ± 0.10
		Water	7.31 ± 0.03	7.71 ± 0.03	Water	6.55 ± 0.01	7.83 ± 0.08
		0.062%	6.19 ± 0.46	<1.5	0.08%	4.95 ± 0.37	1.13 ± 1.96
7418	1	Dried (20 h)	7.74 ± 0.04	7.97 ± 0.02	Dried (20 h)	7.95 ± 0.03	8.26 ± 0.11
		Water	7.35 ± 0.15	7.71 ± 0.06	Water	7.69 ± 0.07	8.11 ± 0.10
		0.062%	7.21 ± 0.21	2.48 ± 4.29	0.08%	6.82 ± 0.11	6.37 ± 0.23
	2	Dried (20 h)	7.64 ± 0.08	8.03 ± 0.19	Dried (20 h)	7.10 ± 0.13	8.08 ± 0.07
		Water	7.24 ± 0.04	7.94 ± 0.28	Water	6.86 ± 0.07	8.06 ± 0.06
		0.062%	7.18 ± 0.18	7.61 ± 0.05	0.08%	3.81 ± 1.36	4.63 ± 0.45
4446	1	Dried (20 h)	7.52 ± 0.15	8.01 ± 0.02	Dried (20 h)	5.54 ± 0.03	8.14 ± 0.03
		Water	6.10 ± 0.13	7.71 ± 0.03	Water	5.62 ± 0.04	8.06 ± 0.03
		0.062%	5.33 ± 0.09	3.69 ± 2.30	0.08%	5.72 ± 0.14	5.76 ± 0.16
	2	Dried (20 h)	7.47 ± 0.07	8.10 ± 0.03	Dried (20 h)	7.74 ± 0.03	8.08 ± 0.03
		Water	6.03 ± 0.03	7.63 ± 0.05	Water	7.00 ± 0.05	8.10 ± 0.03
		0.062%	5.88 ± 0.22	<1.5	0.08%	5.72 ± 0.12	4.50 ± 0.85

*Numbers are the average of triple determinations. SD is standard deviation from the triplicate measurements.

†Strains were grown and spot inoculated in TSB with 1% glucose with or without 5% NaCl. Two trials have been carried out independently.

‡No bacterial cells were detected after disinfection by the plate counts. Hence, log₁₀ < 1.5 is due to the detection limit of the method of 30 CFU coupon⁻¹.

Discussion

In this study, we developed two model systems that allowed quantification of disinfectant sensitivity and hence, comparison of strain susceptibility to disinfectants. Different standards are available to test the efficacy of disinfectants (e.g. Anon 1997, Arnold *et al.* 2006). However, our purpose was to compare strain susceptibility in a true quantitative manner rather than

to compare the efficacy of different disinfectants and we therefore developed set ups that could be used for this purpose. The planktonic *L. monocytogenes* cells were dissolved in 0.9% NaCl because we hypothesize that this will mimic the conditions of the cells after cleaning and rinsing and before disinfection. We adjusted to the same OD₄₅₀-value for planktonic bacteria to obtain the same biomass, and we introduced a standardized biological substrate load for attached bacteria to ensure

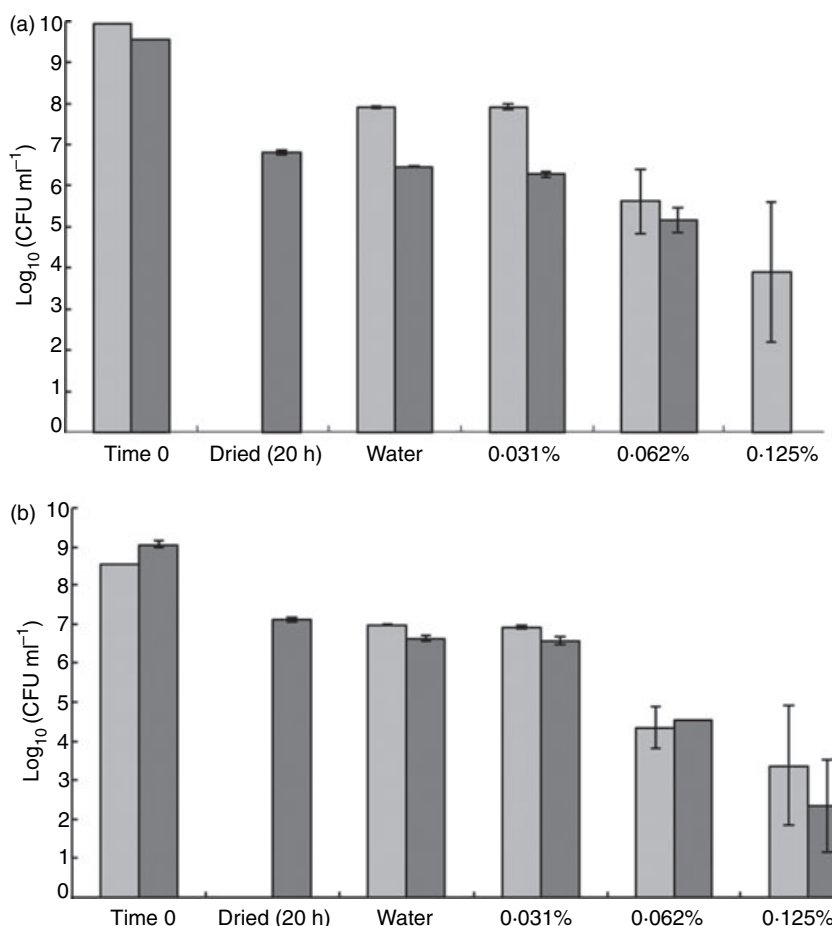


Figure 3 Number of *Listeria monocytogenes* N53-1 bacterial cells after 20 min disinfection of planktonic (light grey) and attached (dark grey) cells with Incimaxx DES. The experiments were performed with planktonic cells re-suspended in 10-fold less volume (a) and planktonic cells re-suspended in the same volume (b). The attached cells were up-concentrated in both experiments. Columns are average of triplicate determinations. Error bars are based on standard deviations from the triplicate measurements.

that the differences among strains were caused by the disinfectant and not by neutralization of the disinfectant by differences in the amount of biological load. We showed that strains of *L. monocytogenes* in our model systems do not differ in a systematic manner in disinfectant tolerance and, as important, attachment does not appear to *per se* protect the bacteria against disinfectants.

In the model with attached bacteria, a high initial number was needed because sensitivity cannot be compared if all bacterial cells are killed by the disinfectant. More bacteria attached to a coupon placed horizontally than to coupons placed in the vertical position. This could be due to gravitation and precipitation of the bacterial cells. However, as the coupons are rinsed before enumeration of the bacteria, the higher number does indeed represent attached organisms. Chavant *et al.* (2004) found that renewal of the growth media increased the number of *L. monocytogenes* adhering to stainless steel coupon and we similarly found that renewal of the medium increased the number of attached cells during 1–2 day incubation period. We were as Kim *et al.* (2007) able to obtain a high number of attached bacteria when

they were spotted and allowed to dry on an inert surface, a set up simulating situations where insufficient cleaning and disinfection allows *L. monocytogenes* to survive protected by organic residues.

Often, sensitivity to disinfectants is determined as a concentration measure e.g. MIC (Sundheim *et al.* 1992; Aase *et al.* 2000; Heir *et al.* 2004) or minimum concentration required to a minimum reduction in viable numbers (Aarnisalo *et al.* 2000; Holah *et al.* 2002). These concentration measures are qualitative and can overestimate the difference between strains. Our set up, where bacterial numbers were quantified after disinfection using three different concentrations, allows calibration to a window where an inter-strain comparison is possible. The set up is not intended to evaluate in-use concentrations proposed by the manufacturer but to allow more fundamental studies of inter-strain differences.

We hypothesized that the remarkable ability of *L. monocytogenes* subtypes to persist in food processing environments for years (Holah *et al.* 2002; Wulff *et al.* 2006; Keto-Timonen *et al.* 2007) could be related to a decreased susceptibility to disinfectants used in the food industry. Our collection of *L. monocytogenes* representing

both persistent and presumed nonpersistent strains did not differ in their sensitivity to two disinfectants, the acidic Incimaxx DES and the alkaline Triquart SUPER. Our results are not similar to findings by Aase *et al.* (2000) that found increased tolerance to both benzalkonium chloride and ethidium bromide in *L. monocytogenes* isolates that had persisted in a fish processing plant for more than 4 years. However, other studies have not been able to link persistence and tolerance to disinfectants (Earnshaw and Lawrence 1998; Holah *et al.* 2002; Heir *et al.* 2004). These studies used different suspension tests and one may hence speculate whether a different pattern of sensitivity tolerance would be revealed by targeting attached bacteria. However, based on the present study, in which both planktonic and attached bacteria were exposed to disinfectants, we find it unlikely that persistent *L. monocytogenes* as a group are inherently more tolerant to disinfectants.

We found initially, as have other studies, that a higher concentration of disinfectant was needed to eliminate attached *L. monocytogenes* as compared with planktonic bacterial cells (Frank and Koffi 1990; Norwood and Gilmour 2000; Stopforth *et al.* 2002; Robbins *et al.* 2005; Folsom and Frank 2006). In the cited studies, different set ups have been used for the disinfection of planktonic and attached cells, respectively, leading to differences in initial cells number and presence of biological material. As biomass and presence of biological material influence the efficiency of disinfectants, it cannot be concluded that if the higher concentration required to eliminate attached bacteria as compared with suspended bacteria is due to a higher biological load introduced by the set up or to increased tolerance of the attached bacteria *per se*. Frank and Koffi (1990) and Stopforth *et al.* (2002) both reported that a higher concentration was needed for the elimination of attached bacteria, but the susceptibility of detached *L. monocytogenes* cells to sanitizer was equal to that of cells in suspension. When we carefully calibrated the models to include equal organic material (substrate and bacteria) and equal volumes and concentrations of disinfectants, *L. monocytogenes* was equally sensitive to disinfectants whether attached or suspended. Together, this indicates that the higher tolerance of attached bacteria to disinfectants is due to one or more of the following factors: (i) protection from organic material, (ii) decreased possibility for the disinfectant to penetrate the cell or (iii) cell-changes only present when the cell is attached.

In this study, growth with 5% NaCl increased the number of attached bacteria on stainless steel coupons significantly at 25°C as has been seen for plastic surfaces (Jensen *et al.* 2007). NaCl increased the tolerance of planktonic cells to Incimaxx DES but not to Triquart SUPER. The increasing tolerance could be because of

changes on the cell surface protecting to Incimaxx DES but not to Triquart SUPER. Some strains may become more hydrophilic when grown in the presence of 5% NaCl (Bereksi *et al.* 2002), which might protect the cell to Incimaxx DES, containing primarily acetic acid and hydrogen peroxide. In contrast, Triquart SUPER contains quaternary ammonium compounds, a surface active cationic detergent (McDonnell and Russell 1999) and EDTA, which may not be affected by the more hydrophilic cell surface caused by NaCl. The surface attached *L. monocytogenes* were not protected against the disinfectants by the addition of NaCl to the growth medium. However, the set up for attachment (spotting on steel) was different from the microtitre well assay in which the pronounced aggregation was observed (Jensen *et al.* 2007).

Incimaxx DES was more effective to planktonic cells as compared with Triquart SUPER, when comparing the concentration necessary for elimination of *L. monocytogenes* bacterial cells. However, the efficiency of Incimaxx DES was to a higher degree influenced by the presence of increased biological material leaving the two disinfectants almost equally effective against spot inoculated, dried bacterial cells. The concentrations used in this study for disinfection of planktonic and spot inoculated, dried cells are lower as compared with recommend in-use concentrations of 1–5% for 5 min.

We demonstrated during the model development that *L. monocytogenes* survives very well 20 h of drying when protected by biological material and presence of 5% NaCl. This was observed for different *L. monocytogenes* strains and is not a unique trait of the persistent subtypes. Rodriguez *et al.* (2007) found that from a mixed *L. monocytogenes* biofilm grown on stainless steel and equilibrated over saturated salt solutions of 94%, 75%, 58%, 33% relative humidity for 24 h, around 10^6 CFU cm⁻² were recovered. However, CFU cm⁻² was not determined before saturation. Ongoing work in our lab (Vogel *et al.* 2007) has demonstrated that *L. monocytogenes* can survive longer than a month at 15°C at low humidity and the survival is remarkably better in media as compared with desiccation in buffer and is enhanced by the presence of NaCl. This emphasizes the importance of sufficient cleaning because the organic material in the food industry very likely contains NaCl. Presence of organic material not only neutralizes the action of the disinfectant but also enables *L. monocytogenes* to survive drying.

As different strains of *L. monocytogenes* were equally tolerant to disinfectants, other factors must explain their ability to persist. In a food processing plant, a heterogeneous microbiota will be present and the resident microbiota may have an impact on the survival of attached *L. monocytogenes* (Carpentier and Chassaing 2004). This co-existence could favour the persistent subtypes because

of better competition or cohabitation with the environmental culture as compared with presumed nonpersistent RAPD-types and is a very interesting area for future research.

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Paper 2

Kastbjerg, V.G., Nielsen, D.S., Arneborg, N. and Gram, L.

Response at the single cell and population level of *Listeria monocytogenes* to disinfection stress monitored by measurements of intracellular pH and viable counts.

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Accepted

1 **Response at the Single Cell and Population Level of *Listeria monocytogenes* to**
2 **Disinfection Stress Monitored by Measurements of Intracellular pH and Viable**
3 **Counts[☼]**

4
5 Running title: Single cell disinfection response of *Listeria monocytogenes*

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19 ☼ Part of the results have been presented at a poster session at the 95th IAFP annual meeting in
20 Columbus, Ohio, USA, August 3-6, 2008.

1 **Abstract**

2 *Listeria monocytogenes* has a remarkable ability to survive and persist in food production
3 environments. The purpose of the present study was to determine if cells in a population of *L.*
4 *monocytogenes* differ in sensitivity to disinfection agents as this could be a factor explaining
5 persistence of the bacterium. In situ analyses of *Listeria monocytogenes* single cells were performed
6 during exposure to different concentrations of the disinfectant Incimaxx DES to study a possible
7 population sub-division. Bacterial survival was quantified with plate counting and disinfection
8 stress at single cell level by measuring intracellular pH (pHi) over time with Fluorescence Ratio
9 Imaging Microscopy.

10 pHi values were initially 7-7.5 and decreased in both attached and planktonic *L.*
11 *monocytogenes* during exposure to sublethal and lethal concentrations of Incimaxx DES. The
12 response of the bacterial population was homogenous; hence, subpopulations were not detected.
13 However, pre-growth with NaCl protected the planktonic bacterial cells during disinfection with
14 Incimaxx (0.0015%) since pHi was higher (6-6.5) for the bacterial population pre-grown with NaCl
15 as compared to cells grown without NaCl (pHi = 5-5.5) ($P < 0.05$). The protective effect of NaCl
16 was reflected by viable counts at a higher concentration of Incimaxx (0.0031%) where the salt-
17 grown population survived better than the population grown without NaCl ($P < 0.05$). NaCl
18 protected attached cells through drying but not during disinfection.

19 This study indicates that a population of *L. monocytogenes* whether planktonic or
20 attached is homogenous with respect to sensitivity to an acidic disinfectant studied on single cell
21 level. Hence a major sub-population more tolerant to disinfectants, and hence more persistent, does
22 not appear to be present.

23

24

1 **Keywords**

2 Intracellular pH (pHi), Fluorescence Ratio Imaging Microscopy (FRIM), Persistence, *Listeria*
3 *monocytogenes*, Disinfection tolerance, NaCl, Viability, Single cell

5 **Introduction**

6 *Listeria monocytogenes* is a food-borne, human pathogen that has a remarkable ability to colonize
7 food processing environments (5, 16, 20, 21, 26, 29). Some *L. monocytogenes* strains can persist for
8 years in food processing plants (11, 14, 20, 27) and specific molecular subtypes can repeatedly be
9 isolated from the processing environment (29) despite being very infrequent in the out door
10 environment (9). This ability to persist has, hitherto, not been linked to any specific genetic or
11 phenotypic trait.

12
13 It has been suggested that persistent *L. monocytogenes* strains may be more tolerant or resistant to
14 cleaning and especially disinfectants used in the food industry. Aase et al. (1) found increased
15 tolerance to both benzalkonium chloride and ethidium bromide in *L. monocytogenes* isolates that
16 had persisted for more than four years; however other studies have not been able to link persistence
17 and tolerance to disinfectants (6, 10, 11, 13). We recently compared disinfection sensitivity of
18 persistent and presumed non-persistent *L. monocytogenes* strains using viable counts and did not
19 find the latter group more sensitive to the two disinfectants Triquart SUPER and Incimaxx DES as
20 compared to persistent strains (13). However, we found that for all subtypes of *L. monocytogenes*,
21 growth with NaCl increased the tolerance of planktonic *L. monocytogenes* to Incimaxx DES,
22 whereas spot inoculated, dried *L. monocytogenes* were not protected by NaCl against disinfection.

23

1 There is no doubt that *L. monocytogenes* will be completely inactivated at the disinfectant
2 concentrations recommended for use in the food industry, however, the efficiency of the
3 disinfectant is very much influenced by presence of organic material being inactivated by presence
4 of food debris. Hence, it is likely that the bacterial cell in a food production environment may be
5 exposed to concentrations at sub-lethal level. It is currently not known if treatment with a sub-lethal
6 concentration of disinfectant affects the entire bacterial population or only attacks a fraction of the
7 cell population, leaving another fraction of cells unaffected. In case of the latter, some bacterial
8 cells may be able to survive the disinfection treatment. The potential presence of such tolerant sub-
9 populations could, ultimately, ensure that the genome is propagated leading to persistence.

10

11 The presence of a more tolerant sub-population can be determined on single cell level. Flow
12 cytometry is a rapid method useable for measurements at the single cell resolution (22), however, it
13 cannot follow the same single cells over time. Optical microscopy combined with microfluidic
14 devices that allow measurement of growth of single cells is a useful technique (2) and in situ
15 analyses of the physiological condition of single cells is the fluorescence ratio imaging microscopy
16 (FRIM) technique represents another elegant approach (25). Fluorescence ratio imaging microscopy
17 enables studies of dynamic changes with high sensitivity and on single cell level in important
18 physiological parameters e.g. intracellular pH (pHi). *Listeria* maintains its pHi within a narrow
19 range of 7.6-8 at extracellular pH (pHex) values of 5.0 to 8.0 (4, 25), and at pHex of 4.0 with the
20 presence of glucose (23). It is believed that viable cells need to maintain a transmembrane pH
21 gradient with their pHi above the pHex, and failure to maintain pHi homeostasis indicates that the
22 bacterial cell is severely stressed and ultimately leads to loss of cell viability. FRIM has been used
23 to determine pHi of *L. monocytogenes* after exposure of osmotic and acid stress (7, 23). Also, the
24 dissipation of the pH gradient in *L. monocytogenes* after exposure to different bacteriocins has been

determined with FRIM (4, 12). Hornbæk et al. (12) found that treatment with subinhibitory concentrations of leucocin and nisin gave rise to two subpopulations; one consisting of cells with a dissipated pH gradient (ΔpH) and the other consisting of cells that maintained ΔpH which could indicate phenotypic heterogeneity.

The aim of the present study was to investigate the physiological effects of the disinfectant Incimaxx DES at sublethal and lethal concentrations on single cells and population level of a persistent *L. monocytogenes* strain to study a possible sub-division of sensitivity in the population. We also addressed the potential protective effect of NaCl against disinfection and compared sensitivity in a population of planktonic and attached bacteria. We applied the in situ technique FRIM and compared the pH_i measurements with the traditional viable count method.

Materials and Methods

Bacterial strains and media. *Listeria monocytogenes* strain N53-1 was isolated from equipment in a fish smoke house (29). It belongs to the RAPD type 9 cluster, a molecular subtype that has been persistent for many years in several fish-processing industries (28). Stock cultures were stored at -80 °C in 4% (wt/vol) glycerol, 3 % (wt/vol) tryptone soya broth (TSB; CM129, Oxoid; Basingstoke, UK), 2 % (wt/vol) skim milk powder and 0.5% (wt/vol) glucose. The bacteria were cultivated on brain heart infusion (BHI) agar (CM0225, Oxoid, Basingstoke, UK supplemented with 1.5% agar) at 30 °C and kept at 4 °C for maximum a month. Subsequent cultures were prepared in TSB supplemented with glucose to a final concentration of 1% (wt/vol) plus in some trials addition of NaCl to a final concentration of 5% (wt/wt). The bacteria were grown overnight at 30 °C, diluted 1000 times and grown at 30 °C for 22 h.

1 **Fluorescence labelling of bacterial cells.** Fluorescent labelling of *L. monocytogenes* N53-1 with
2 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Molecular Probes, Inc. Eugene,
3 Oreg.) was carried out as follows: Bacterial cells were harvested by centrifugation at 14,000 *X g* for
4 2 min and re-suspended in sterile-filtered (pore size, 0.22 μm ; GP Express Membrane Filter;
5 Millipore, Bedford, Mass.) citric acid-phosphate-buffer solution (pH 7.0) containing 0.37 %
6 (wt/vol) citric acid monohydrate and 2.93 % (wt/vol) Na_2HPO_4 (19). Glucose and the fluorophor,
7 CFDA-SE, were added to obtain final concentrations of 10 mM and 10 μM , respectively, and the
8 cells were incubated at 30 °C for 30 min. Cells were harvested by centrifugation at 14000 *X g* for 2
9 min. For studies of planktonic bacteria, the pellet was resuspended and adjusted in 0.9% NaCl
10 (wt/vol) with 10 mM glucose to an optical density at 600 nm (OD_{600}) of 0.4 to standardize the
11 biomass. For studies of spot inoculated and dried *L. monocytogenes*, the cells were resuspended and
12 adjusted in sterile growth media to $\text{OD}_{600} = 1.0$. Bacteria were kept under these conditions for no
13 longer than 2 h.

14
15 **Immobilisation chambers for planktonic bacteria.** Planktonic *L. monocytogenes* were
16 immobilised on a glass surface essentially as described by Shabala et al. (24). Briefly, a glass cover-
17 slip was cleaned with a 70% ethanol, 1% HCl mixture, rinsed with sterile MilliQ-water and dried.
18 Poly-L-lysine solution (0.1% wt/vol aqueous solution, P 8920, Sigma Diagnostics, St. Louis, USA)
19 was used to attach cells to the glass surface. One drop ($\sim 30 \mu\text{l}$) of the poly-L-lysine solution was
20 applied to the cover-slip and left for about 5 min to dry. Subsequently, a CoverWellTM perfusion
21 chamber gasket (C18128, Molecular Probes, Inc. Eugene, Oreg.) were attached and the cover-slip
22 with chamber were mounted on a platform designed for the purpose.

23

1 **Measurement of pHi of individual bacterial cells.** The pHi of individual *L. monocytogenes* N53-1
2 bacterial cells was measured by fluorescence ratio imaging microscopy (FRIM) as described by
3 Guldfeldt and Arneborg (8). This set-up consisted of an inverted epifluorescence microscope
4 (Axiovert 135 TV; Zeiss, Birkerød, Denmark) equipped with a Zeiss Fluar X 100 objective
5 (numerical aperture, 1.3), a dichroic mirror (510 nm), and an emission band-pass filter (515-565
6 nm). Bacterial cells were excited at 488 and 435 nm with exposure time of 1000 ms by a
7 monochromator equipped with a 75-W xenon lamp (Monochromator B; TILL Photonics GmbH,
8 Planegg, Germany). To minimize photo bleaching of the stained cells, a 10% neutral-density filter
9 was inserted between the optical fiber and the microscope for the experiments done with the
10 planktonic bacteria, whereas a 2.5% neutral-density filter were used for the experiments with spot
11 inoculated and dried bacteria. Fluorescence emission was collected with a cool charge-coupled
12 device camera (CCD) camera (Coolsnap FX, Photometrics, Roper Scientific) and images were
13 analyzed by using the Metavue 6.1 software (Molecular Devices, Dowington, PA). Regions were
14 defined around approximately 40 to 50 individual cells for each experiment by using Metavue. A
15 region near but without the cell was subtracted for each cell as background. The ratio value
16 ($R_{488/435}$) for each cell examined was obtained by dividing the fluorescence intensity at 488 nm (pH
17 sensitive wavelength) with the fluorescence intensity at 435 nm (a pH insensitive wavelength). The
18 ratio values were transformed to pHi values by using a calibration curve. To construct this, ethanol
19 (70%, vol/vol) was added to CFDA-SE stained *L. monocytogenes* N53-1 cells for 5 min to
20 permeabilise the membrane irreversibly. Subsequently, the bacterial cells were harvested by
21 centrifugation at 14000 *X g* for 2 min and resuspended in citric acid – phosphate buffers having pH
22 values ranking from 5.0 to 7.7 (19). The calibration curve used for spot inoculated, dried *L.*
23 *monocytogenes* N53-1 was constructed for bacteria permeabilised and resuspended in TSB having
24 pH values ranking from 5.0 to 9.5.

1

2 **Preparation of disinfectant solutions.** The disinfectant Incimaxx DES (Ecolab Denmark ApS,
3 Valby, Denmark), commonly used in the food industry as disinfectant and for decalcification,
4 contains a mixture of peroxyacids and hydrogen peroxide as active ingredients. Disinfectant
5 solutions were prepared by dilution in sterilized, demineralized water to obtain concentrations
6 where differences in pH_i were seen. Disinfectant solutions were prepared in 10 times the strength of
7 desired treatment right before use for testing planktonic bacteria and in desired concentrations for
8 disinfection of spot inoculated bacteria.

9

10 **Exposure of planktonic *L. monocytogenes* N53-1 to Incimaxx DES.** At time zero, 60 µl of a
11 disinfection solution was mixed thoroughly with 540 µl of bacterial cell suspension. Seventy µl
12 were transferred to the CoverWell™ perfusion chamber gasket, and microscopic images were
13 acquired every 2.5 min for 20 min in at least three defined positions. Sterile demineralized water
14 was used as control. Also, to determine the number of viable *L. monocytogenes* during disinfection,
15 40 µl of cell suspension were transferred to 160 µl Dey-Engly (DE) neutralising broth (281910,
16 Difco, BD, Sparks, France) every 5 min for 20 min. This was done in duplicate. Preliminary
17 experiments showed that DE broth neutralized disinfectants also in higher concentrations of
18 disinfectants than used, and that viability of *L. monocytogenes* was unaffected when cells were
19 suspended in DE broth (13). Bacterial numbers were determined by surface plating on BHI agar
20 plates that were incubated at 37 °C for 2 days. The complete experiment was repeated twice. Based
21 on a set of preliminary experiments of disinfection sensitivity, *L. monocytogenes* were exposed to
22 Incimaxx DES concentrations of 0.0062%, 0.0031% and 0.0015% (vol/vol). To monitor the
23 extracellular pH (pH_{ex}) in the bacteria-disinfection mixture, pH were determined every 5th min for

20 min. Before each experiment, microscopic images were acquired of the bacterial cell suspension of *L. monocytogenes* N53-1 to obtain an initial ($t = 0$) pH_i -value.

Exposure of spot inoculated and dried *L. monocytogenes* N53-1 to Incimaxx DES. Sterile cover slips were placed on a wire screen in a laminar-flow biosafety cabinet. Twenty μ l of CFDA-stained *L. monocytogenes* N53-1 cells suspended in sterile growth media were deposited on each coupon, and dried for 20 hours in the laminar-flow biosafety cabinet. Spot inoculated slips were covered with a CoverWellTM perfusion chamber gasket (C18120; Molecular Probes, Inc. Eugene, Oreg.) and 320 μ l Incimaxx DES were added. Sterilised demineralized water was used as control. To monitor pH_i during disinfection, microscopic images were acquired every 2.5 min for 20 min in three defined positions. To monitor viability of spot inoculated, dried bacteria, cover slips were disinfected in parallel in triple determinations for 20 min. After 20 min of incubation, the coupons were transferred with the disinfectant to 10 ml DE neutralising broth, and bacteria were detached by sonication for 4 min (28 kHz 2 x 150 W sonication bath, Delta 220, Deltasonic Meaux, France) (15), vortexed at maximum speed for 15 s and diluted 10-fold serially. Also, three untreated coupons were transferred directly to DE neutraliser broth and sonicated. Cell numbers were determined by surface plating on BHI agar plates that were incubated at 37 °C for 2 days. The experiment was repeated at least twice. *L. monocytogenes* was exposed to Incimaxx DES, concentrations of 0.062%, 0.031% and 0.015% (vol/vol). Before each experiment, microscopic images were acquired of a chamber with spot inoculated and dried *L. monocytogenes* N53-1 added 0.9% NaCl to obtain an initial pH_i -value. pH of the disinfectants were determined. This equals pH_{ex} in the bacteria-disinfection mixture, as the disinfectant volume used is 16 times the bacterial volume.

Statistical analysis. Statistical comparisons were made between means of pH_i or log₁₀ transformed bacterial counts using Student's *t*-test with a significance level of $P < 0.05$.

Results

Construction of calibration curves. Calibration curves ($R_{488/435}$ versus pH_i) were prepared using ethanol-treated cells of *L. monocytogenes* N53-1 suspended at different pH values in citric acid-phosphate buffer (planktonic bacteria) or TSB-media (attached bacteria) (Fig 1). The two calibration curves differed at the low ratio values and were more uniform at the high ratio values. The probe is very pH sensitive at pH 6.0 to 9.0, while values below pH 6.0 can be difficult to distinguish (Fig. 1). Hence, ratio values less than 1.78 or 1.88 were recorded as pH 5.0 for planktonic and attached bacteria, respectively.

pH_i of planktonic *L. monocytogenes* N53-1 exposed to Incimaxx DES. The viable count and pH_i of planktonic *L. monocytogenes* N53-1 grown in TSB with 1% glucose were determined following 20 min exposure to three different concentrations of Incimaxx DES or to demineralized water as control (Fig 2, 3). pH_i decreased during the 20 min treatment to pH 5 for all three concentrations of Incimaxx DES, indicating that the bacterial cells were stressed (Fig 2). The rapidity in change in pH_i differed between the three Incimaxx DES concentrations. pH_i decreased to less than 5.5 in 5 min of treatment with 0.0062%. The change was slower but similar for 0.0031% and 0.0015% where pH_i decreased to less than 5.5 in 10 min. Demineralized water caused only a minor decrease from pH_i 7.33 ± 0.06 to pH_i 6.75 ± 0.28 during the 20 min treatment. The extracellular pH in the bacteria-disinfection solutions was 4.0 - 4.1 during 20 min with all three concentrations of Incimaxx DES including water (data not shown). This indicates that the decrease in pH_i during disinfection is due to Incimaxx DES and not just an effect of low external pH. The change in pH_i was similar in all

cells measured indicating that the response of the bacterial population was homogenous (Fig 2).

During the early treatment with Incimaxx DES, pH_i of the bacterial cells was distributed over 1-1.5 pH units; however pH_i of the bacterial cells decreased to the same $\text{pH}_i \pm 0.5$ and did not split into sub-populations.

The viable counts remained constant over time when *L. monocytogenes* N53-1 was treated with water, indicating that the minor decrease from $\text{pH}_i 7.33 \pm 0.06$ to $\text{pH}_i 6.75 \pm 0.28$ did not affect viability (Fig 3). Disinfection with 0.0015% Incimaxx DES caused only a minor decrease in cfu/ml after 20 min, and this decrease was not significantly different ($P > 0.05$) from counts of bacteria treated with water. Hence, despite the low pH_i of 5 to 5.5 caused by 0.0015% Incimaxx DES, the stressed bacterial cell were able to recover and grow on agar. A more pronounced reduction in viable cells of *L. monocytogenes* N53-1 was seen during treatment with 0.0031% and especially 0.0062% Incimaxx DES. The counts of bacteria treated with 0.0062% were already after 5 min significantly different ($P < 0.05$) from bacteria treated with water or 0.0015% Incimaxx DES.

The counts of *L. monocytogenes* N53-1 pre-grown with 5% NaCl did not decrease (cfu/ml) when the bacteria were exposed to 0.0015% Incimaxx DES as compared to water. A slight reduction in viable counts was seen for bacteria exposed for 20 min to 0.0031% Incimaxx (Fig 3a and 4a). This protective effect by NaCl was reflected in the pH_i of the bacterial cells, and pH_i of *L. monocytogenes* N53-1 remained at 6-6.5 when treated with Incimaxx DES at 0.0015%. In comparison, the pH_i of 5.5 for bacteria grown without NaCl was significantly lower ($P < 0.05$). The response of the bacterial population when grown with 5% NaCl where homogenous and no subpopulations were detected (data not shown).

pHi of spot inoculated and dried *L. monocytogenes* N53-1 exposed to Incimaxx DES. The response of a bacterial population of *L. monocytogenes* N53-1 grown in TSB with 1% glucose and spot inoculated was homogenous during treatment with Incimaxx DES (Fig 5). However, pH_i of the population was distributed over a broader range of pH values over time as compared to the planktonic population (Fig 2). There was no indication of formation of sub-populations of cells with increased tolerance. pH_i decreased rapidly to less than 5.5 after 5 min of treatment with 0.062% Incimaxx DES (fig 6b). Exposure to 0.031% and 0.015% Incimaxx DES caused the same decline in pH_i from 7.0 to 5.7 during 20 min of treatment. Only a minor decrease in pH_i from 7.0 to 6.5 was seen over time when treated with demineralized water.

The cell numbers of *L. monocytogenes* N53-1 on glass surfaces were equal after treatment with water, 0.015% or 0.031% Incimaxx DES. However, 0.062% Incimaxx DES caused a marked decrease in bacterial numbers (Fig 6a).

Addition of NaCl to *L. monocytogenes* N53-1 caused more cells to survive the drying process, since the cfu/coupon were significantly higher ($P < 0.05$) as compared to *L. monocytogenes* N53-1 grown, spot inoculated and dried in TSB with 1% glucose (Fig 6a and 7a). This indicates that NaCl protects the bacterial cells during drying. Also, this protective effect could be seen from the pH_i measurement during treatment with water, since pH_i of *L. monocytogenes* N53-1 grown and spotted with 5% NaCl was 7.1 after 20 min while it was 6.5 for *L. monocytogenes* N53-1 grown and spotted without NaCl. This indicates that preculture with NaCl enable the bacterial cell to keep pH_i more constant in water. However, when disinfecting *L. monocytogenes* N53-1 dried with 5% NaCl, the number of remaining bacterial cells decreased as rapidly as the non-NaCl grown cells with increasing concentration of Incimaxx DES indicating that pre-culture and spot with NaCl renders

1 the attached, dried bacterial cells more or as sensitive to Incimaxx DES as compared to N53-1
2 grown and spotted without NaCl.

3

4 **Discussion**

5 Some sub-types of *L. monocytogenes* are able to persist in food processing plants for many years
6 (11, 14, 20, 27, 29), and this is a major food safety issue as they constitute a constant reservoir of
7 food product contamination. The seriousness of this is highlighted by a recent (summer 2008)
8 outbreak of listeriosis in Canada that has been caused by meat products contaminated with *L.*
9 *monocytogenes* on two processing lines in a meat factory. The ability to persist has, hitherto, not
10 been linked to any specific genetic or phenotypic trait. It has been hypothesised that persistent *L.*
11 *monocytogenes* strains were less susceptible to disinfection than non-persistent strains; however,
12 most studies have not been able to confirm this hypothesis (6, 10, 11, 13). However, the persistent
13 strains may reside in the processing environment due to the constant maintained tolerant sub-
14 population and such heterogeneity at the single-cell level would be masked in a study of a
15 sensitivity and killing kinetics in the complete bacterial population. In the present study we have for
16 the first time used FRIM to study the physiological effect of disinfectants at the single-cell level by
17 measuring intracellular pH. We did, however, not find indication that sub-lethal concentrations of
18 the disinfectant studied caused division of the population in sub-populations.

19

20 As mentioned, the present study is to our knowledge the first study using measurement of pH_i to
21 detect the appearance of sub-populations during disinfection. The technique detected single cell
22 physiological response to the stress factor at concentrations where the determination of bacterial
23 density (cfu/ml or cfu/coupon) did not reveal any effects. Other studies have followed pH_i over time
24 for exponential and stationary-phase cells of *L. monocytogenes* during exposure to benzalkonium

1 chloride (17). However, in these studies, the pH_i was measured on a population basis using
2 spectrophotometry and single cells could not be followed. In the present study, we did not detect
3 any more tolerant sub-population, neither in the planktonic nor in the attached population, since the
4 decrease in pH_i was very homogenous for the all cells over time. Earlier, the FRIM method has
5 successfully been used to detect subpopulations in *L. monocytogenes* cultures exposed to
6 bacteriocins (12). Similarly, *L. monocytogenes* previously grown on agar plates were
7 heterogeneous with respect to sensitivity to nisin when measuring pH_i, and some of the cells
8 appeared to be more tolerant than others (4).

9

10 A value of pH_i 5.0-5.5 is the lower limit of the sensitivity for CFDA (3) which was also seen from
11 the calibration curves. As the external pH is around 4, the pH_i measurement can be used for
12 information on stress conditions of the cells but not on viability. Another dye with higher sensitivity
13 at lower pH could have been used instead. However, the low pH-sensitive dye would not cover the
14 neutral pH range, leading to loss of information. Instead we combined the pH_i measurement with
15 viability measured as cfu on agar plates.

16

17 We found that growth with a typical food component, NaCl, protected the bacteria against drying
18 and disinfection. When using pH_i as a measure of bacterial response, the protective effect was seen
19 at treatments with 0.0015% Incimaxx, whereas it was reflected by viable counts at a higher
20 concentration of Incimaxx (0.0031%) in accordance with earlier results (13). As pH_i decreased very
21 rapidly to below 5.5 during disinfection with 0.0031% Incimaxx DES, one may speculate if using a
22 probe more sensitive in the lower pH-area could have indicated a protective effect of NaCl. Bacteria
23 that were spot inoculated and dried on surfaces were only protected by NaCl during the drying stage
24 but not when exposed to disinfectants. This is in agreement with our previous data using colony

counts to measure effect of disinfectants (13). A higher concentration of Incimaxx DES was needed to eliminate attached *L. monocytogenes* as compared to planktonic bacteria cells. However, this is more likely due to the higher biological load introduced by the set-up than to increased tolerance of the attached bacteria *per se*.

In the present study, *L. monocytogenes* survived pHi less than 5.5 for more than 10 min as planktonic cells during disinfection with 0.0015% Incimaxx DES, and viability was not significantly different from the viability in water. Shabala et al. (23) measured a pHi of ≤ 5 after 2 hours for *L. monocytogenes* maintained at pHex 3.0 and it remained viable as it recovered immediately and remained constant at pHi 7.3, when returning to pHex 6.0. Hence, the ability of this organism to sustain a low pHi, even though it is critical for many cellular processes, such as DNA transcription, protein synthesis, and enzyme activities, may contribute to the survival of *L. monocytogenes* in acidified environments.

pHi was a more sensitive measure of adverse effects on *L. monocytogenes* than viability (cfu). Thus, a concentration of Incimaxx DES (0.0015%) that did not significantly affect cell counts had a marked effect on pHi (Fig. 3). Similarly, Luppens et al (18) found that the ability to maintain a pH gradient was largely affected by BAC and hydrogen peroxide before a major loss in viability (according to plate counts) was detected. This indicates that antibacterial components may clearly stress bacterial cells even at levels where no effect is seen on viable counts. Measurements of pHi allow an on-line indication of the physiological status of bacterial cells and can be used to follow both individual bacterial cells and a population of bacteria. Due to the high sensitivity of the pHi measurement it is useful for determination of sub-inhibitory concentrations of disinfectants i.e. concentrations that do stress the bacteria by decreasing pHi but do not affect viability. This is highly

1 useful for further studies of the impact of sub-inhibitory stress on *L. monocytogenes* in relation to
2 fx. gene-expression and virulence. Furthermore, the method may be useful for studying sub-
3 inhibitory concentrations of antibiotics.

4
5 In conclusion we have shown that the response of a persistent strain of *L. monocytogenes* whether
6 planktonic or attached is homogenous on single-cell level to an acidic disinfectant; hence sub-
7 populations do not appear. It is not likely that the persistent strain of *L. monocytogenes* survives in
8 the production environment due to presence of a more tolerant subpopulation. The pH_i
9 measurement is useful for determination of sub-inhibitory concentrations of disinfectants and is
10 relevant for further studies of the impact of sub-inhibitory stress on *L. monocytogenes*.

13 **Acknowledgements**

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17 assistance.

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1 **Figure legends**

2 **FIGURE 1.** Relationship between $R_{488/435}$ of the individual cells of *Listeria monocytogenes* N53-1
3 and pH_i . The pH_i was equilibrated to pH_{ex} by incubating preparations with 70% ethanol and
4 dissolving in different pH values in buffer (A) or TSB-media (B). The ratio values are averages
5 based on at least 40 single cells. The error bars indicate standard deviations.

6
7 **FIGURE 2.** Change in the pH_i of *Listeria monocytogenes* N53-1 grown in TSB with 1% glucose
8 during disinfection with (A) water (control), (B) 0.0015%, (C) 0.0031%, and (D) 0.0062%
9 Incimaxx DES. Each point shows the pH_i of a single cell.

10
11 **FIGURE 3.** Viable counts (A) and pH_i (B) of *Listeria monocytogenes* N53-1 grown in TSB with
12 1% glucose and resuspended in 0.9% NaCl with 10 mM glucose during treatment with water (◆),
13 0.0015% (■), 0.0031% (▲) and 0.0062% (—) Incimaxx DES. The viable counts are average of
14 duplicate determinations. Error bars are based on standard deviations from the duplicate
15 determinations. pH_i are averages based on at least 40 single cells from fig 2. and error bars indicate
16 population variation. Arrows indicate that cfu were under the detection limit in the plating assay (10
17 cfu/ml). The results are representative of two independent experiments.

18
19 **FIGURE 4.** Viable counts (A) and pH_i (B) of *Listeria monocytogenes* N53-1 grown in TSB with
20 1% glucose and 5% NaCl, resuspended in 0.9% NaCl with 10 mM glucose during treatment with
21 water (◆), 0.0015% (■), 0.0031% (▲) and 0.0062% (—) Incimaxx DES. The viable counts are
22 average of duplicate determinations. Error bars are based on standard deviations from the duplicate
23 determinations. pH_i are averages based on at least 40 single cells and error bars indicate population

variation. Arrows indicate that cfu were under the detection limit in the plating assay (10 cfu/ml).

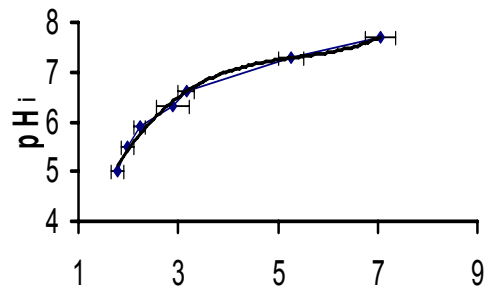
The results are representative of two independent experiments.

FIGURE 5. Change in the pHi of *Listeria monocytogenes* N53-1 grown, spot inoculated and dried in TSB with 1% glucose during disinfection with (A) water (control), (B) 0.015%, (C) 0.031%, and (D) 0.062% Incimaxx DES. Each point shows the pHi of a single cell.

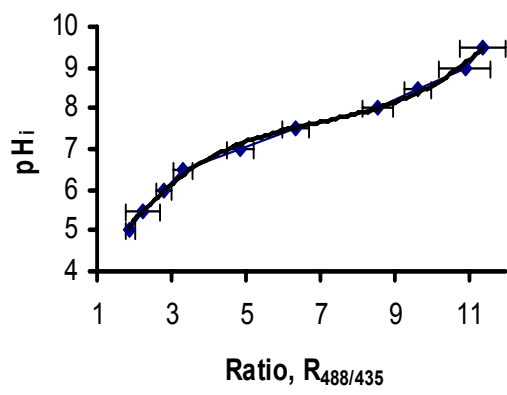
FIGURE 6. Viable counts (A) and pHi (B) of *Listeria monocytogenes* N53-1 grown, spot inoculated and dried in TSB with 1% glucose during treatment with water (◆), 0.015% (■), 0.031% (▲) and 0.062% (—) Incimaxx DES. The viable counts are average of triplicate determinations. Error bars are based on standard deviations from the triplicate determinations. pHi are averages based on at least 40 single cells and error bars indicate population variation. The results are representative of two independent experiments.

FIGURE 7. Viable counts (A) and pHi (B) of *Listeria monocytogenes* N53-1 grown, spot inoculated and dried in TSB with 1% glucose and 5% NaCl during treatment with water (◆), 0.015% (■), 0.031% (▲) and 0.062% (—) Incimaxx DES. The viable counts are average of triplicate determinations. Error bars are based on standard deviations from the triplicate determinations. pHi are averages based on at least 40 single cells and error bars indicate population variation. Arrows indicate that cfu were under the detection limit in the plating assay (100 cfu/coupon). The results are representative of two independent experiments.

A



B



1

2 **FIGURE 1**

3

4

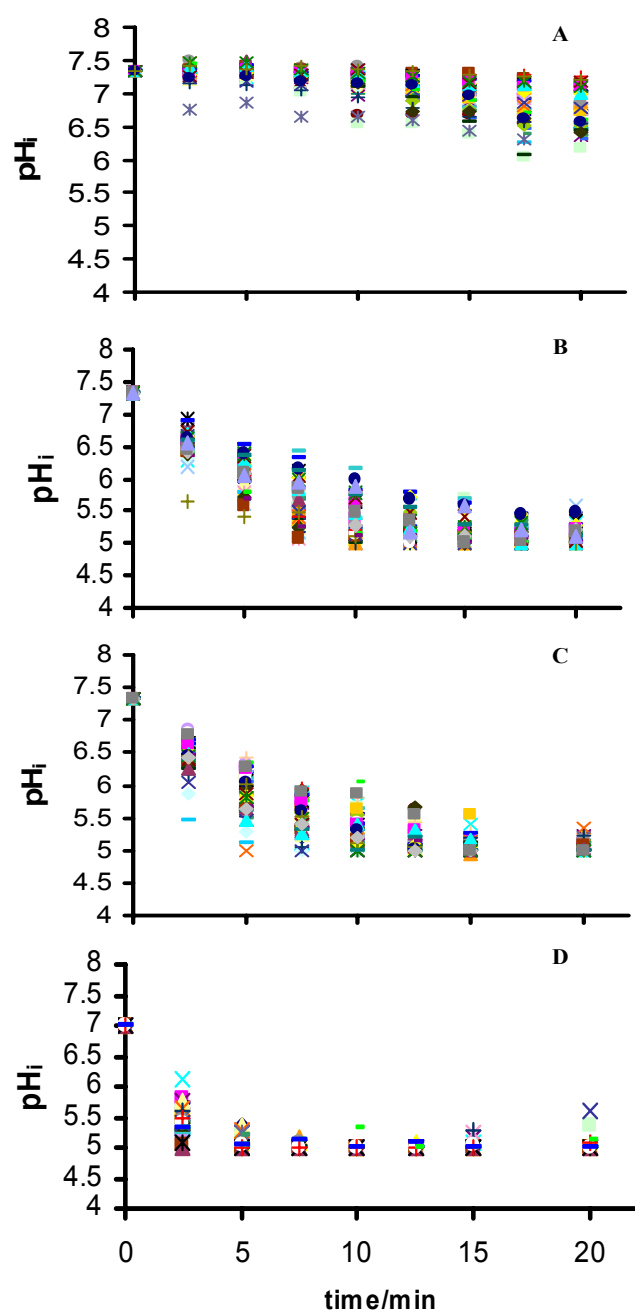


FIGURE 2

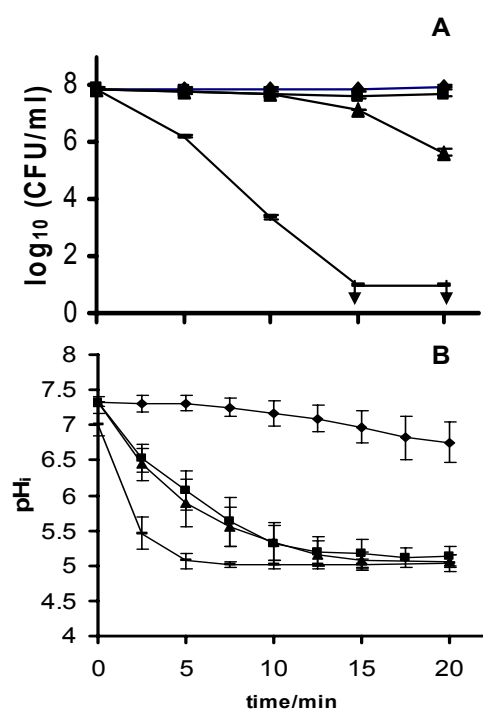


FIGURE 3

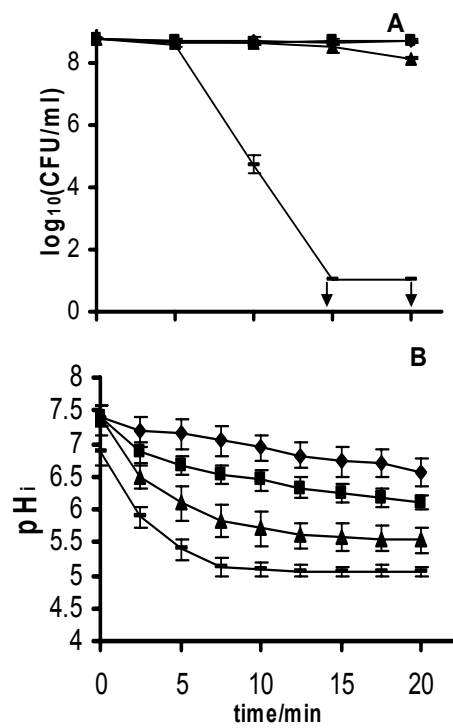


FIGURE 4

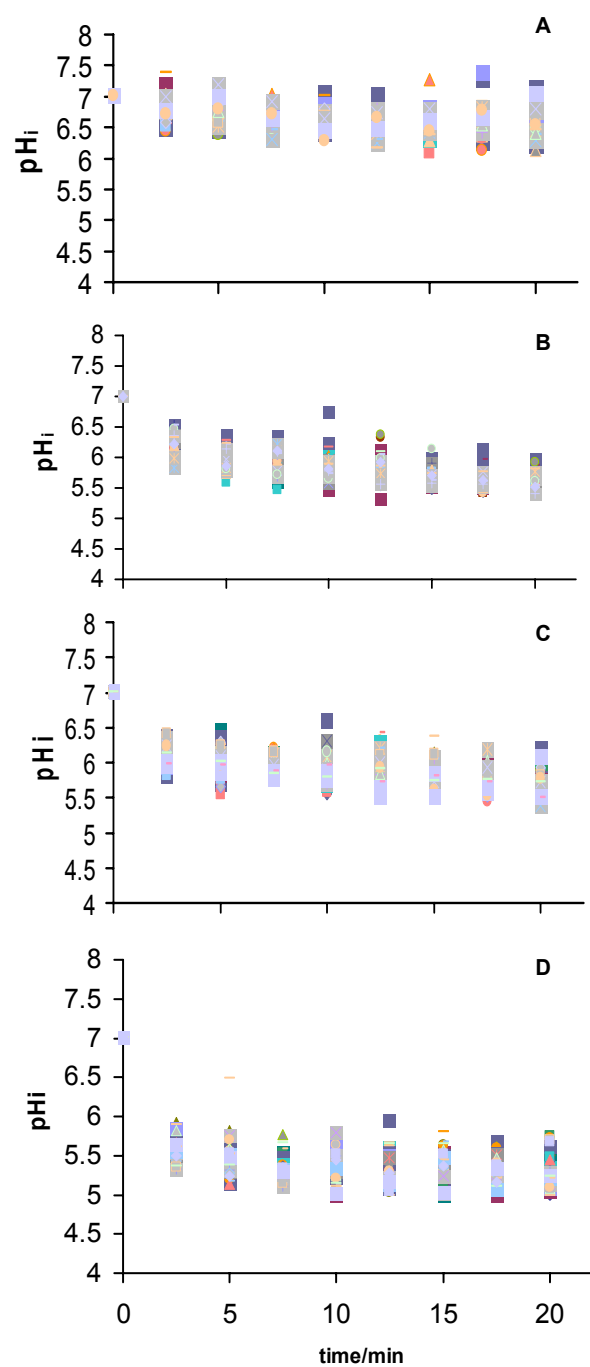


FIGURE 5

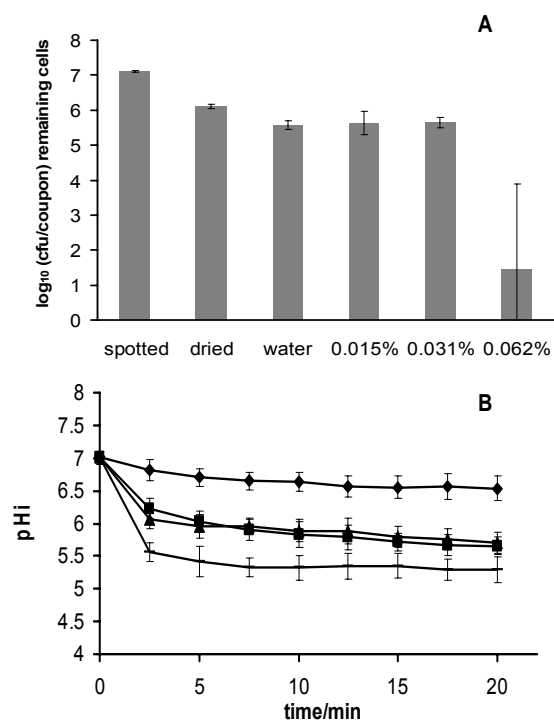


FIGURE 6

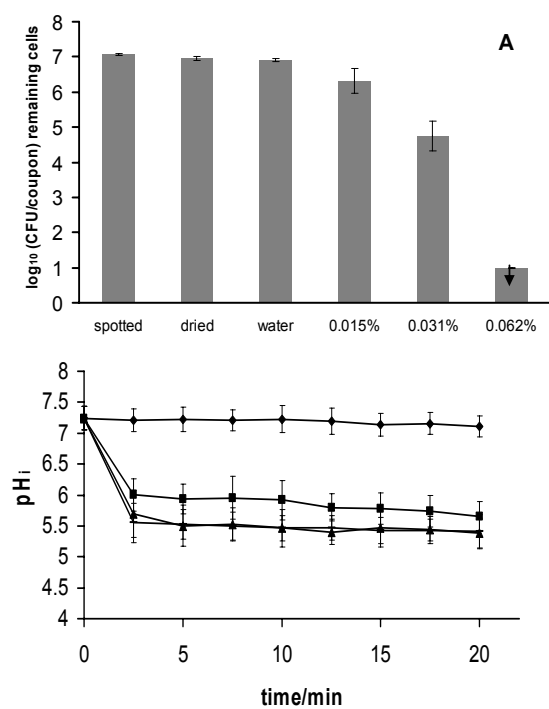


FIGURE 7

Paper 3

Kastbjerg, V.G., Larsen, M.H., Gram, L. and Ingmer, H.

Sublethal concentrations of common disinfectants influence virulence gene expression in
Listeria monocytogenes.

Applied and Environmental Microbiology.

Submitted

1 **Sub-lethal concentrations of common disinfectants influence virulence gene**
2 **expression in *Listeria monocytogenes***

3

4 Running title: Disinfectants affect virulence gene expression in *L. monocytogenes*

5

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18

1 **Abstract**

2 *Listeria monocytogenes* is a food-borne human pathogen that causes listeriosis, a relative rare
3 infection with a high fatality rate. The regulation of virulence gene expression is influenced by
4 several environmental factors, and the aim of the present study was to determine how disinfectants,
5 used routinely in the food industry, affect the expression of different virulence genes in *L.*
6 *monocytogenes* when added in sub-lethal concentrations. An agar-based assay was developed to
7 screen the effect of disinfectants on virulence gene promoter expression and was validated at the
8 transcriptional level by Northern blot analysis. Eleven disinfectants representing four different
9 groups of active components were evaluated in this study. Disinfectants with the same active
10 ingredients did cause a similar effect on gene expression. Peroxy- and chlorine compounds reduced
11 the expression of the virulence genes and quaternary ammonium compounds (QAC) induced the
12 expression of the virulence genes. In general, a disinfectant had similar effect on the expression on
13 all four virulence genes examined. Northern blot analyses confirmed the down-regulation of *prfA*
14 and *inlA* expression by Compound 1 (a peroxy compound) and the up-regulation by Compound 7 (a
15 QAC) in *L. monocytogenes* EGD. Hence, sub-lethal concentrations of disinfectants routinely used
16 in the food industry affect virulence gene expression in the human pathogen *L. monocytogenes*, and
17 the effect depend on the active components of the disinfectant. Further studies are needed to
18 elucidate if the change in virulence gene expression induced by the disinfectants have impact on
19 virulence or other biological properties such as antibiotic resistance.

21 **Keywords**

22 Virulence gene expression, *Listeria monocytogenes*, disinfectants, sub-inhibitory concentrations,
23 *lacZ*-fusion

24

1 **Introduction**

2 *Listeria monocytogenes* is a food-borne, facultative intracellular pathogen that can cause invasive
3 listeriosis in immunocompromised individuals, pregnant women, infants and the elderly. The
4 disease is relatively rare; 0.3 cases per year per 100,000 inhabitants EU, but it is associated with a
5 high fatality rate (25-30%) (11).

6

7 *L. monocytogenes* can cross the intestinal barrier, the blood-brain barrier and the placental barrier,
8 and it invades and replicates in both phagocytic and non-phagocytic cells (47). The ability to
9 invade, survive and multiply within eukaryotic cells is determined by a number of chromosomal
10 genes of which most are found on the 9-kb pathogenic island known as LIPI-1 (*Listeria* pathogenic
11 Island 1), but also other places on the chromosome. These virulence genes encode products
12 involved in adherence to and internalization by the host cell (*inlA*, *inlB*, *inlC*), escape from the
13 vacuoles (*hly*, *plcA*, *plcB*), intracellular replication (*htp*), and cellular movement (*actA*). The major
14 transcriptional factor which regulates the expression of these virulence genes including its own
15 transcription is the 27 kDa polypeptide, PrfA (5, 8, 12, 18, 27, 32).

16

17 The regulation by the PrfA protein on virulence gene expression is dependent on concentration and
18 activity of the protein but also on promoter configuration of the virulence gene promoters. The PrfA
19 protein facilitates specific binding to its target site, the so-called PrfA box that partially overlaps the
20 promoter region, and affinity is weakened when this target sequence diverges from the perfect
21 palindromic sequence (17, 44, 49).

22

23 The regulation of PrfA and virulence gene expression is influenced by several environmental
24 factors. One example is the temperature-dependent control of translation of *prfA* messenger, which

1 is only processed at 37 °C and not at 30 °C (22, 26). Another example is the repression of virulence
2 genes expression in response to high concentrations of fermentable carbohydrates (35), and a third
3 example is the induction of virulence gene expression observed when *L. monocytogenes* is grown
4 with activated charcoal, due to the absorption of an autorepressor (13, 40). Also less well-described
5 physico-chemical parameters affect virulence gene expression. Nutrients stress conditions limiting
6 growth induce gene expression of *prfA*, *plcA*, *hly* and *actA* (3, 45), and high osmolarity induce
7 expression of *hly* (37). On the other hand, low pH (pH 6 – 6.7) repressed *hly* expression (2).
8
9 *L. monocytogenes* is frequently detected in the food-processing environment where it has a
10 remarkable ability to reside (7, 29, 34, 38, 50). An efficient cleaning and disinfection process is
11 essential in preventing contamination of food-products with *L. monocytogenes* during processing.
12 However, the disinfection process is not always adequately performed or organic debris inactivates
13 the disinfectant; hence the bacteria may only be exposed to sub-lethal concentrations and survive.
14 *L. monocytogenes* that reside in food processing environments may adapt to disinfectants after
15 repeated exposure (1, 46); however, few studies have investigated how low concentrations of
16 disinfectants affect the physiology of *L. monocytogenes* at gene expression level. Recently, we
17 found that exposure of *L. monocytogenes* to sub-lethal concentrations of a disinfectant stressed the
18 cell, measured as a decrease in intracellular pH (24), and Ryan et al. (42) found that the *sigB* gene
19 was up-regulated in presence of quaternary ammonium compounds and SDS, common components
20 of industrial cleaning agents. Hence, an element in assessing the risk from *L. monocytogenes*
21 contaminating a food product is to determine if food environment stress factors such as sub-lethal
22 concentrations of disinfectant affect expression of virulence genes in *L. monocytogenes*.
23

The aim of the present study was to determine how disinfectants, used routinely in the food industry, affect the expression of different virulence genes in *L. monocytogenes* when added in sub-lethal concentrations. An agar-based assay was developed and applied for screening the effect of disinfectants on virulence gene promoter expression and was validated at the transcriptional level by Northern blot analysis.

Materials and methods

Bacterial strains, media and culture conditions. *Listeria monocytogenes* EGD and Δ prfA were obtained from Werner Goebel (Biozentrum). Strains of *L. monocytogenes* EGD with *lacZ* fusions to the *hly*, *plcA*, *prfA* and *inlA* promoters in the vector pTCV-lac were from the laboratory collection at LIFE (25). Stock cultures were stored at -80 °C in 15% (wt/vol) glycerol. The bacteria were cultivated on brain heart infusion (BHI) agar (CM0225, Oxoid, Basingstoke, UK supplemented with 1.5% agar) at 37 °C for 24-48 h. One colony were cultured in BHI broth and incubated with shaking (180 rpm) at 37 °C overnight, diluted 1000 times and grown at 37 °C, 180 rpm for 18 h. Kanamycin were added when required at a final concentration of 50 µg/ml.

Preparation of disinfectant solutions. Eleven disinfectants commonly used in the food industry were tested in this study (Table 1). The disinfectants represent four different groups of active compounds. Disinfectants were prepared in two-fold dilutions in sterilized, demineralised water to obtain concentrations where inhibitions zones with appropriate size (i.e. zones did not merge) were seen in the agar-assay (described below).

1 **Agarbased screening assay.** An agar based assay was developed on the basis of an existing assay
2 in *Staphylococcus aureus* (A. Nielsen, K.F. Nielsen, T.O. Larsen and H. Ingmer, in preparation).
3 BHI agar and charcoal-supplemented BHI (BHI-AC, BHI with addition of 0.013% or 0.025%
4 activated charcoal) was melted and tempered to 44 °C. X-gal (150 µg/ml) and kanamycin (50
5 µg/ml) were added. One ml of outgrown bacterial culture with promoter:*lacZ*-fusion, diluted 1000
6 times in 0.9% NaCl, was mixed thoroughly with 25 ml of media in a petri dish (diameter 9 cm), and
7 the plates were dried in a LAF-bench for 45 minutes. Seven wells were made in each plate and 30
8 µl of disinfectant in different concentrations were added to each well. Water was used as control.
9 The plates were incubated at 37 °C for 48 hours. Each disinfectant was tested in at least two
10 independent experiments with all strains containing promoter-*lacZ* fusions. In each experiment, all
11 strains were screened in BHI and BHI-AC. Strains containing plasmids *pprfA-lacZ*, *phly-lacZ* and
12 *pinlA-lacZ* were screened in 0.013% BHI-AC, while the strain containing plasmid *pplcA-lacZ* were
13 screened in 0.025% BHI-AC in order to further induce promoter activity (see below).

14
15 **Construction of promoter-*lacZ* fusions in a Δ prfA strain.** To study if the response of the
16 different virulence gene promoters were influenced by the PrfA protein, the *lacZ*-fusions were
17 transformed into the Δ prfA *L. monocytogenes* EGD strain. Listerial plasmid DNA was purified
18 from strains containing plasmid *phly-lacZ*, *pinlA-lacZ* and *pplcA-lacZ* using the Qiaminiprep kit
19 (Qiagen). The plasmid isolation procedure was modified by incubating the cell suspension in p1
20 buffer containing lysozym (9mg/ml) for 1 h at 37 °C. Competent Δ prfA cells were prepared as
21 described by Park and Stewart (39) with slight modifications. Briefly, 3 ml of an overnight culture
22 were cultured in 150 ml BHI with 0.5 M sucrose at 37 °C with shaking (200 rpm) until reaching
23 OD₆₀₀ = 0.2. Penicillin G, 10 µg/ml, was added and the incubation continued for a further 2 h (100
24 rpm). Cells were harvested (3000 x g, 10 min, 4 °C) and washed three times with sucrose

1 electroporation buffer (1 mM Hepes pH 7.0; 0.5 M sucrose). The final cell pellet was resuspended
2 in 500 µl ice-cold glycerol solution (1mM Hepes; 0.5M sucrose; 15% glycerol) and stored at -80 °C
3 prior to use. Cells, 40 µl and 1 µl plasmid DNA was mixed and electroporation were performed (25
4 µF, 2,5 kV, 200 ohm). After the pulse, 1 ml prewarmed BHI (37 °C) were added, mixed with the
5 cells, and transferred to 15 ml BHI. After 3 h incubation at 30 °C with shaking (150 rpm), cells
6 were spread on BHI plates with kanamycin (5 µg/ml).

7
8 **RNA extraction and Northern hybridization.** An outgrown culture of *L. monocytogenes* EGD
9 was diluted 100 times in BHI and grown to OD₆₀₀ = 0.4. The culture was split and centrifuged at
10 3000 x g for 10 min, and the pellet re-suspended to the same OD in BHI-AC broth (0.2% charcoal)
11 with a concentration of 0.25 % and 0.125 % Compound 1 or in BHI broth with a concentration of
12 0.0031% and 0.0016 % Compound 7, and 60 ml was transferred to a 300 ml Erlenmeyer flask.
13 Demineralised water was used for the control cultures. Cells were grown at 37 °C with shaking (130
14 rpm). Samples were taken after 15, 30, 60 and 180 min of growth with the disinfectant for RNA-
15 extraction. RNA was stabilized by the addition of two volumes RNA protect[®] (Qiagen), and
16 bacterial cells were harvested by 5000 x g for 10 min and stored at -80 °C. Bacterial numbers were
17 determined by surface plating on BHI agar plates after 0, 60 and 180 min of growth. BHI agar
18 plates were incubated at 37 °C overnight. To extract RNA, the bacterial cells were lysed using the
19 Fast Prep FP120 instrument (BIO101, ThermoSavent) for 45 s at speed 6. The cells were kept on
20 ice for one minute and then lysed again. The treatment and cooling on ice were repeated three
21 times. Total RNA was extracted from the cells using the RNeasy mini kit (Qiagen) according to the
22 manufacturers directions. Analysis of transcripts was performed as described by Frees et al. (15)
23 with slight modifications. Briefly, total RNA was quantified with Nanodrop (Nanodrop 2000,
24 Thermo Fisher Scientific), and 5 µg of RNA of each preparation were loaded onto a 1% agarosegel

and separated in 10 mM sodium phosphate buffer. RNA was transferred to a positively charged nylon membrane (Amersham HybondN, GE healthcare) by capillary blotting. Hybridization probes were generated by PCR from chromosomal DNA of *L. monocytogenes* EGD using specific primers for the *prfA* gene (1F: 5'TAA CCA ATG GGA TCC ACA AG-3' and 1R: 5'TGC TAA CAG CTG AGC TAT GTG-3') and the *inlA* gene (1,5F: 5'ATC GAT GGA GTG GAA TAC TT-3' and 1,5R: 5' GTG CCT ATA TCT TTT AAC TGG TTA C-3'). Probes were labeled with [³²P]-dCTP using the Ready-to-go DNA-labelling beads (Amersham Biosciences). RNA from at least two independent experiments was analyzed.

Results

Screening of disinfectants in agar-assay. To determine how different disinfectants affect the expression of virulence genes in *L. monocytogenes*, we designed an agar-based screening assay in which virulence gene expression is monitored as β -galactosidase production from *L. monocytogenes* derivatives carrying *lacZ* fused to each of the known virulence factor genes *hly*, *plcA*, *prfA* and *inlA* (25). In order to be able to monitor reductions in virulence gene expression, we chose to use a growth medium that supports a low level of virulence gene expression as is the case with BHI and BHI-AC.

When we incorporated the *L. monocytogenes prfA-lacZ*-strain in BHI or BHI-AC agar plates, the reporter strain turned strong blue indicating that the *prfA* gene was induced during growth in BHI and that charcoal did not visually induce the *prfA* promoter further (Fig. 1). The *lacZ* gene of the *inlA*- and *hly-lacZ* fusions was induced in BHI but addition of charcoal caused an even more intense blue colour indicating an induction of the promoters by charcoal. In contrast, addition of activated charcoal was necessary to obtain a visible *lacZ* expression of the *plcA-lacZ* fusion, and the amount

1 of charcoal needed to obtain a sufficient blue colour was two-fold higher as compared to the other
2 strains (Fig 1).
3
4 After having confirmed that differences in virulence gene expression could be monitored in agar
5 plates, disinfectants were added to wells formed in the agar, and each of the *lacZ* fusion strains were
6 examined in BHI and BHI-AC agar. Disinfectants were added in inhibitory concentrations, and for
7 all strains the size of the inhibition zones decreased as expected with decreasing concentration of
8 disinfectant (Fig 1). Also, each well represents a screening of disinfectant concentrations as it
9 dilutes outward from each well. The size of the inhibition zones decreased when charcoal was
10 added confirming that the efficacy of the disinfectant is influenced by the amount of organic
11 material. For some disinfectants, white colonies were seen outside of the inhibition zones indicating
12 that this disinfectant in sub-lethal concentrations reduced the expression of the virulence gene (Fig
13 1A). For other disinfectants, colonies outside the inhibition zones were of a more intense blue
14 colour as compared to the colonies further away from the inhibition zones indicating that this
15 disinfectant in sub-lethal concentrations induced the expression of the virulence gene (Fig 1B). No
16 inhibition or effect on gene expression was seen when water was added to the well (control).
17
18 In total, eleven disinfectants representing four groups of active components were evaluated in the
19 present study (Table 1). They are all commonly used in the food industry and supplied by different
20 manufacturers. Disinfectants with the same active ingredients did in general cause a similar effect
21 on gene expression. Peroxy- and chlorine compounds reduced the expression of the virulence genes
22 and quaternary ammonium compounds (QAC) induced the expression of the virulence genes.
23 However, for Compound 2, white colonies were seen around the inhibition zone followed by an
24 intense blue zone, which could indicate that the disinfectant in a sub-lethal concentration caused

1 reduction in the expression of the virulence gene but in a lower concentration caused induction of
2 the virulence gene expression.

3
4 In general, a compound had the same effect on all four virulence genes. However, with Compound
5 4 reduction was followed by induction on the *inlA* gene promoter whereas reduction alone was seen
6 with the other three virulence gene promoters. Similarly, Compound 6 induced the *prfA*, *inlA* and
7 *hly*-promoters, but had no visible effect on the *plcA*-promoter.

8
9 **Screening of Δ prfA *lacZ*-fusion strains.** Since many of the virulence genes in *L. monocytogenes*
10 are controlled by PrfA, we determined if the effect caused by the different disinfectants on virulence
11 gene expression was dependent of PrfA. To study this, the plasmids containing the reporter gene
12 fusions (*pplcA-lacZ*, *pinlA-lacZ*, and *phly-lacZ*) were transformed into the *prfA* deletion mutant.

13
14 The three Δ prfA promoter-*lacZ* transformants were all screened in the agar assay. Expression of
15 *inlA* was induced in BHI, and addition of charcoal increased the expression also in the absence of
16 PrfA. However, there was no visible expression of *plcA* or *hly* in the *prfA* deletion strains during
17 growth in BHI or BHI-AC. Neither could growth of the two strains in diluted BHI (1:2, 1:4, 1:8)
18 agar (1.5%) or diluted TSB (1:7) agar (1.5%) with or without 0.013% charcoal induce the
19 promoters. Hence, only the Δ prfA *pinlA-lacZ* strain could be screened with the disinfectants in our
20 agar assay.

21
22 The influence of the eleven disinfectants on gene expression in Δ prfA *pinlA-lacZ* strain were
23 determined. All disinfectants had the same effect on this strain as in the *pinlA-lacZ* strain, which
24 contains the *prfA* gene (Table 1). When comparing the intensity of the background color as well as

the blue induction zones or white colonies in the reduction zones, no clear difference was seen between the $\Delta prfA$ *pinLA-lacZ* strain and the *pinLA-lacZ* strain. This indicates that the up- and down-regulation of the *inlA* promoter by disinfectants was not primarily caused by PrfA.

Northern blot analysis of virulence gene expression in the presence of disinfectant. To study if the effect of disinfectants on the virulence gene promoter was indeed a true gene induction or repression effect, we determined the effect of sub-lethal concentrations of disinfectants on the transcript level of the *prfA* and *inlA* genes. Northern blot analyses were performed with the wildtype *L. monocytogenes* EGD and the two disinfectants, Compound 1 and Compound 7 that down- and up-regulate gene expression in the agar-assay, respectively. Both disinfectants were tested in sub-lethal concentrations (Fig. 2).

When *L. monocytogenes* EGD was grown with 0.25% and 0.125% Compound 1, a marked down-regulation was seen on transcript level for *prfA* and *inlA* as compared to EGD grown with water (Fig. 3). The *plcA-prfA* transcript of 2.1 kb was markedly down-regulated both at 15, 30, 60 and 180 min of growth with both concentrations of Compound 1 (lane B, E, H, K and lane C, F, I, L) as compared to the control (lane A, D, G, J) (Fig 3a). Similarly, the monocistronic *prfA* transcripts of 0.8 and 0.9 kb were down-regulated as compared to control trials. The downregulation was more pronounced in trials with 0.25% Compound 1 as with 0.125% Compound 1. Both transcripts *inlA* (2.9 kb) and *inlAB* (5 kb) were detected after 15 min of growth in the control, whereas only the *inlA* transcript was detected during further growth (fig 3b). However, only very weak transcripts of *inlA* were detected in *L. monocytogenes* EGD grown with both concentrations of Compound 1.

Also, up-regulation of *prfA* and *inlA* in *L. monocytogenes* EGD grown in BHI with Compound 7 were seen on transcript level with the Northern blot analysis (Fig. 4, data not shown). The monocistronic *prfA* transcripts were markedly up-regulated after 15, 30 and 60 min growth with 0.0031% Compound 7 (lane B, E, H) as compared to the control (lane A, D, G). Also, growth with 0.0016% Compound 7 up-regulated *prfA* after 60 min. The transcript at 2.1 kb was only weakly expressed, since *L. monocytogenes* was grown in BHI broth without addition of charcoal. Only a weakly monocistronic *inlA* transcript was detected. However induction could be seen after 30 min growth with 0.0031% Compound 7 (data not shown).

All together, the Northern blot analyses confirmed the observations obtained with the agar assay.

Discussion

In the present study, we have developed an agar-based assay on the basis of an existing assay intended to isolate fungal compounds that reduce virulence gene expression in *S. aureus* (A. Nielsen, K.F. Nielsen, T.O. Larsen and H. Ingmer, in preparation). Similarly, our assay can be used to study how different compounds affect virulence gene expression in *L. monocytogenes*. In the screening assay, the expression is measured as β -galactosidase activity of virulence gene promoters fused to a *lacZ* gene. However, this relates very well to transcript level as confirmed by Northern blot analyses. The assay has high sensitivity, since it detects differences in expression on transcript level, but it is not quantitative. In the present study, we used the assay to determine the effect of different disinfectants routinely used in the food industry on the gene expression of four virulence genes, but the assay could also be used for screening of other compounds and the influence on other (virulence) genes in *L. monocytogenes*.

1 In general, disinfectants with the same active ingredients had similar effect on virulence gene
2 expression. Peroxy- and chlorine compounds reduced the virulence gene expression, and QAC
3 induced the expression of the virulence. Ryan et al. (42) found that two QAC,
4 benzalkoniumchloride (BC) and cetylpyridinium chloride (CPC) in sub-lethal concentrations,
5 induced expression of *sigB* in *L. monocytogenes* measured by reverse transcriptase PCR, hence
6 QAC may affect a number of bacterial behaviors. The difference in gene expression is not due to
7 differences in pH *per se*, since disinfectants with peroxy compounds have a low pH, whereas the
8 disinfectants with chlorine and QAC compounds generally have a high pH. Others have determined
9 the effect of sub-inhibitory concentrations of commercially disinfectants on virulence in other
10 pathogens. Disinfectants including QAC reduced virulence factors when measured on phenotypic
11 level in *Pseudomonas aeruginosa* and *Streptococcus agalactiae* (19, 20, 30), benzyl alcohol
12 induced expression of an operon involved in biofilm formation in *Staphylococcus epidermis*, and
13 peracetic acid induced some and down regulated other of the virulence-related genes in *S. aureus*
14 when studied by transcriptomics (6, 36).

15
16 PrfA is transcribed both as monocistronic *prfA* from its own two promoters and as a *prfA-plcA*
17 bicistron from the *PplcA* (4, 16, 32). Basal amounts of PrfA are made from the monocistronic *prfA*
18 transcripts and these stimulate the transcription of the *prfA-plcA* bicistron. In our Northern blot
19 analysis no transcript was detected at 2.1 kb in trials with Compound 1 and when *L. monocytogenes*
20 EGD was grown in BHI, which can be due to a too low basal amount of monocistronic *prfA*. The
21 increase caused by Compound 7 was not sufficient to induce the transcription of the *prfA-plcA*
22 bicistron. Interestingly, the effect on promoter activity and transcription level indicates that the
23 disinfectants, even in non-lethal levels, interact with the transcription process directly or indirectly.
24 This could be through interaction with the promoter-region or the RNA polymerase, or with the (so-

far unidentified cofactor that are proposed to be involved in the) allosteric transition of PrfA that is necessary to stimulate the *PplcA* and other virulence promoters (41, 48, 49).

It is well-known that PrfA regulates the expression of several virulence genes in *L. monocytogenes* including *hly*, *inlA*, *plcA* and *prfA*. However, activation by PrfA is more efficient at promoters which possess a perfectly symmetrical PrfA box as *PplcA* or *Phly* than at promoters which have substitutions such as *PinLAB* (44). Also, some virulence genes have more than one promoter. *plcA* is transcribed from one PrfA-dependent promoter, and *hly* and *inlA* are transcribed from three and four promoters, respectively. Two *hly* promoters are PrfA-dependent and one PrfA-independent, whereas only one of the *inlA* promoters is PrfA-dependent (9, 10, 28, 33). This might lead to the different dependency on PrfA seen in this study, where only the *PinlA* was sufficiently induced during growth in the Δ PrfA strain to produce a visible amount of β -galactosidase. In the screening assay, PrfA did not visibly affect the different disinfectants' effects on the *PinlA*. This indicates that the modulation on *inlA* expression by disinfectants is not dependent on PrfA concentration. However, an interaction by PrfA on *PinlA* cannot be excluded by this assay.

To our knowledge, this is the first study showing that disinfectants routinely used in the food industry, in sub-lethal concentrations affect virulence gene expression in the human pathogen, *L. monocytogenes* and that the effect depends on the active component in the disinfectant. Further studies are necessary to elucidate if these differences have biological importance. One may hypothesise that sub-lethal concentrations of disinfectants could affect virulence of strains exposed to sub-lethal concentrations of disinfectants. This would be highly relevant in risk analysis of the use of disinfectants; especially in settings where the route of the pathogen from disinfectant exposure to the host is shorter as it is in food processing settings.

1
2 Also, recently concerns have been expressed about the potential effect of biocide exposure on
3 development of resistance to disinfectants or antibiotics. Resistance to disinfectants is believed to be
4 a relatively rare event because most disinfectants are often complexes of antimicrobials that
5 inactivate multiple cell targets (31). However, sub-lethal concentrations of disinfectants can select
6 for antibiotic resistance. In *Salmonella* Typhimurium a QAC showed the highest selectivity for
7 variants with reduced susceptibility of different antibiotics (23). This variant had increased level of
8 *acrB*, a marker for efflux leading to increased sensitivity to antibiotics. Also, induction of efflux
9 pumps caused by biocides has been detected in other pathogens (14, 21, 43). Similarly, the increase
10 in virulence gene expression detected with some disinfectants could be general on more genes in *L.*
11 *monocytogenes*, thereby lead to a selection of antibiotic resistance.

12

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Table 1. Effect of different disinfectants on expression of different virulence genes of *Listeria monocytogenes*.

Type of disinfectant	Designation	Concentration	Down(-) or up(+) regulation of gene				
			prfA-fusion	plcA-fusion	inlA-fusion	ΔprfA inlA-fusion	hly-fusion
Peroxy compounds	Compound 1	1.25% - 40%	-	-	-	-	-
	Compound 2	0.31% - 10%	-/+	-/+	-/+	-/+	-/+
Chlorine	Compound 3	3.13% - 100%	-	-	-	-	-
	Compound 4	3.13% - 100%	-	-	-/+	-/+	-
	Compound 5	3.13% - 100%	-	-	-	-	-
Triclosan, ethanol	Compound 6	0.19% - 6%	+	no	+	+	+
Quaternary ammonium compounds	Compound 7	0.0063% – 0.2%	+	+	+	+	+
	Compound 8	0.0063% – 0.2%	+	+	+	+	+
	Compound 9	0.0063% – 0.2%	+	+	+	+	+
	Compound 10 (neutral pH)	0.0063% – 0.2%	+	+	+	+	+
	Compound 11 (acid pH)	0.0063% – 0.2%	+	+	+	+	+
	Compound 11 (alkaline pH)	0.0063% – 0.2%	+	+	+	+	+

+ represents a induction in the expression of the virulence gene

- represents reduction in the expression of the virulence gene

-/+ represents reduction followed by induction in the expression of the virulence gene

No: neither induction nor reduction in the expression of the virulence gene

1 **Figure legends**

2

3 **Figure 1.** (A) *lacZ*-fusions with *Listeria monocytogenes* *prfA* (I), *plcA* (II), *inlA* (III) and *hly* (IV)
4 promoters diluted 1000 times and cast in BHI (*prfA*,) or BHI-AC (*plcA*, *inlA* and *hly*). Compound 1
5 is added in decreasing concentration in well 1-6. (B) *lacZ*-fusions with *prfA* (I), *plcA* (II), *inlA* (III)
6 and *hly* (IV) promoters diluted 1000 times and cast in BHI (*prfA*, *inlA*, *hly*) or BHI-AC (*plcA*).
7 Compound 7 is added in decreasing concentration in well 1-6.

8

9 **Figure 2.** Growth of *Listeria monocytogenes* EGD in (A) BHI broth with (♦) water (control), (■)
10 0.0031% and (▲) 0.0016% Compound 7 and in (B) BHI-AC (0.2% charcoal) broth with (♦) water
11 (control), (■) 0.25% and (▲) 0.125% Compound 1.

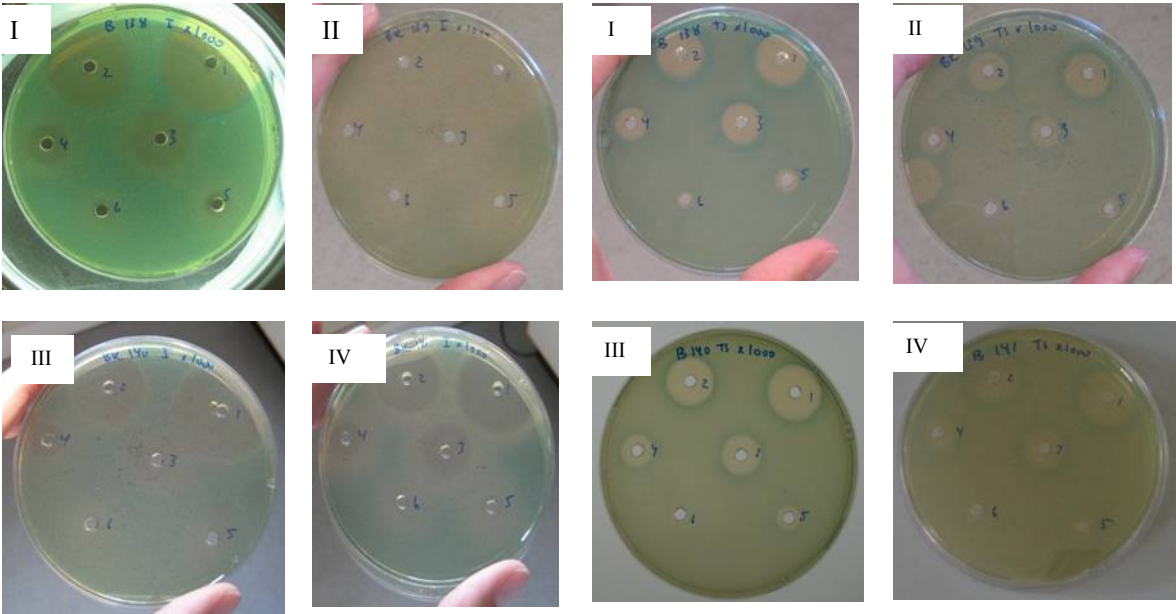
12

13 **Figure 3.** *Listeria monocytogenes* *prfA* (A) and *inlA* (B) transcription measured by Northern blot
14 using RNA isolated from *L.monocytognes* EGD grown in BHI-AC (0.2% charcoal) for 15 min
15 (lanes A, B, C), 30 min (lanes D, E, F), 60 min (lanes G, H, I) and 180 min (J, K, L) with water
16 (control) (lanes A, D, G, J), 0.125% (lanes B, E, H, K) or 0.250 % Compound 1 (lane C, F, I, L).
17 Arrows indicates the sizes of the *plcA-prfA* (2.1 kb), *prfA* (0.8 and 0.9 kb), *inlAB* (5 kb) and *inlA*
18 (2.9 kb) transcripts.

19

20 **Figure 4.** *prfA* transcription measured by Northern blot using RNA isolated from *Listeria*
21 *monocytognes* EGD grown with 15 min (lanes A, B, C), 30 min (lanes D, E, F), 60 min (lanes G, H,
22 I) and 180 min (J, K, L) with water (control) (lanes A, D, G, J), 0.0031% (lanes B, E, H, K) or
23 0.0016% Compound 7 (lane C, F, I, L). Arrows indicates the sizes of the *plcA-prfA* (2.1 kb) and
24 *prfA* (0.8 and 0.9 kb) transcripts.

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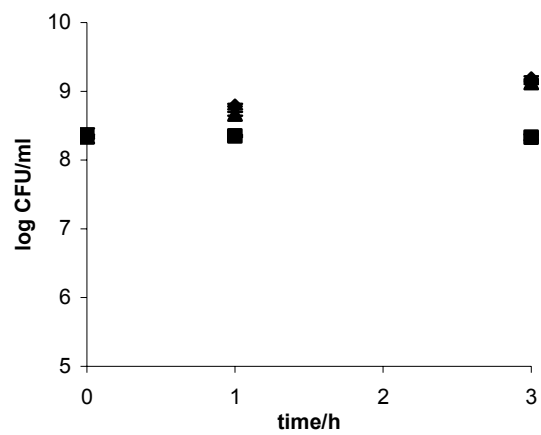
A **B**

4
5

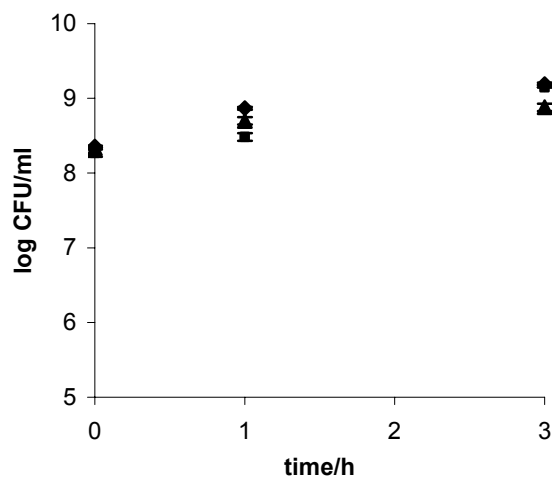
Figure 1

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A



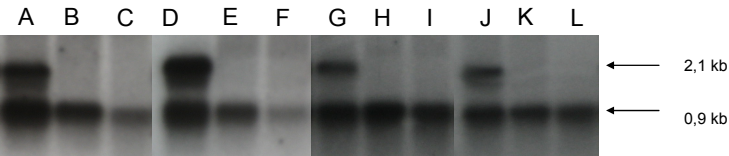
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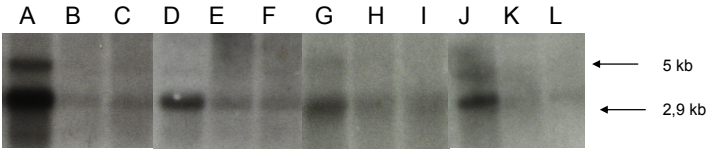
2 **Figure 2**

1

2 **A**

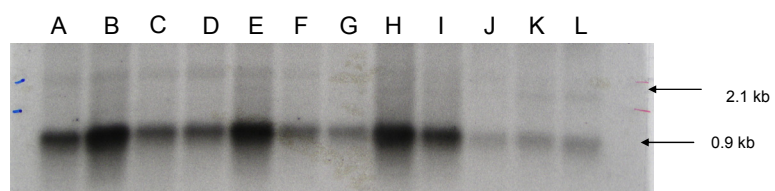


3 **B**



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5 **Figure 3**



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3 **Figure 4**

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